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(54) Title: APPARATUS AND METHOD FOR CAPTURING PARTICLES WITH SURFACE EXPOSURE OF ANIONIC PHOSPHOLIPIDS FROM BIOLOGICAL FLUIDS

(57) Abstract

An affinity filter and method of using same, effective in capturing and thereby removing particles characterized by surface exposure of anionic phospholipids present in a biological fluid, particularly blood or blood–derived products. Examples of other biological fluids include semen, cerebrospinal fluid, urine and mucous. The affinity filter includes a body formed with an inlet and an outlet, including a solid support and an anionic–phospholipid binding compound linked to the solid support. The positively charged anionic–phospholipid binding compound serves for specifically binding the particles characterized by surface exposure of anionic phospholipids and thereby removing the particles from the biological fluid, and particularly from blood or blood–derived products, for example in order to prepare the blood or blood–derived product for transfusion into a subject.

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APPARATUS AND METHOD FOR CAPTURING PARTICLES WITH SURFACE EXPOSURE OF ANIONIC PHOSPHOLIPIDS FROM BIOLOGICAL FLUIDS

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to an apparatus and method for capturing particles characterized by surface exposure of anionic phospholipids from biological fluids such as blood and blood-derived products and, more particularly, to an apparatus and method for removing microemboli from transfused blood or blood-derived products prior to transfusion thereof and/or microemboli generated during extracorporeal circulation employed during various medical and surgical procedures, including heart surgery.

The present invention is particularly useful in the prevention of thromboembolic events during certain surgical and medical procedures, in particular during extracorporeal circulation, e.g., during cardiac surgery, or during blood transfusion.

Extracorporeal circulation is now routinely used during heart surgery. The prevalence of these surgical procedures is currently very high. However, there is an increasing appreciation of long-term complications associated with these procedures, of which neurological complications are specifically prominent. The incidence of cerebral stroke after coronary artery bypass operation is between 0.8 % and 5.0 %. Neuropsychological dysfunction of variable duration and degree is reported in up to 80 % of patients (Jacobs A, et al., Stroke 1998;29:660-667).

It is also recognized now that these impairments are, in part, due to showers of microemboli created and spread during the cardiopulmonary bypass. These microemboli are comprised, in part, of cell debris, apoptotic cells, apoptotic bodies and microparticles generated from activated platelets, as well as from other cell types.

The widespread lodging of these microparticles in the capillaries of various organs, notably in the central nervous system, is currently considered a cardinal factor in the pathogenesis of organ damage and dysfunction following the surgical procedure (Moody DM, et al., Ann Neurol 1990;28:477-486; Blauth CI, Ann Thorac Surg 1995;59:1300-1303, Clark RE, et al., J Thorac Cardiovasc Surg 1995;109:24-258).

Certain filtering procedures are used to minimize this risk. They consist mainly of placing a size-based filter (e.g., 40 μm filter) on the arterial line. This measure has been shown to improve the neurological outcome of the cardiopulmonary bypass (Pugsley W, et al., Stroke,

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1994;25:1393-1399), though it does not eliminate the microemboli phenomenon (Jacobs A, et al., Stroke 1998;29:660-667).

One of the emerging concepts regarding these microparticles is their procoagulant activity, with stimulation of thrombin formation (Nieuwland R, et al., Circulation 1997;96:3534-3541). This procoagulant effect has been attributed to the exposure of anionic phospholipids, mainly phosphatidylserine (PS), on the membrane surface.

Normally, anionic phospholipids are restricted to the inner leaflet of the plasma membrane. This distribution, hereby designated cell membrane lipid asymmetry (CMLA) is constantly and actively maintained in most eukaryotic cells (Zwaal RFA & Schronit AJ, Blood 1997;89:1121-1132).

However, as further detailed hereinunder, during certain physiological and mainly pathological states, notably platelet activation, platelet microparticle generation and apoptosis, there is loss of CMLA (Chang CP et al., J Biol Chem 1993;268:7171-7178; Rosing J, et al., Blood 1985;65:319-326; Van den Eijnde SM, et al., Cell Death Diff 1997;4:311-316).

The consequent surface exposure of anionic phospholipids renders the membrane a potent catalytic surface for formation of clotting factor complexes, i.e., the tenase and prothrombinase complexes (Mann KG, et al., Blood 1990;76:1-16).

Indeed, it has been shown that microparticles generated during cardiopulmonary bypass are characterized by both enhanced surface exposure of anionic phospholipids, and highly procoagulant activity (Nieuwland R, et al., Circulation 1997;96:3534-3541).

The lodging of the showers of microemboli in the microvasculature during cardiopulmonary bypass may therefore be associated with multifocal small vessel thrombosis and occlusion.

Therefore, there is a need for novel methods for detection, capture and removal of microparticles, such as microemboli, from the systemic circulation during cardiopulmonary bypass.

Similar considerations also imply to routine blood transfusion. Storage of blood and blood-derived products, e.g., red blood cell (RBC) concentrate and platelet-enriched plasma, has been consistently shown to be associated with increased amounts of damaged and senescent cells, characteristically associated with surface exposure of anionic phospholipids (Sestier C et al., C R Acad Sci III 1995;318:1141-1146), which may putatively render these cells highly procoagulant, again through the loss of CMLA.

It is therefore desirable to detect and remove these cells and cell-derived particles, thus

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improving the quality and potentially increasing the life-span of the blood-derived product.

Apoptosis is another major situation in which CMLA loss takes place. Apoptosis is an intrinsic program of cell self-destruction or "suicide", which is inherent in every eukaryotic cell. In response to a triggering stimulus, cells undergo a highly characteristic cascade of events of cell shrinkage, blebbing of cell membranes, chromatin condensation and fragmentation, culminating in cell conversion to clusters of membrane-bound particles (apoptotic bodies), which are thereafter engulfed by macrophages (Boobis AR, et al., Trends Pharmacol. Sci. 10:275-280, 1989; Bursch W, et al., Trends Pharmacol. Sci. 13:245-251, 1992). Loss of CMLA is quite a universal phenomenon in apoptosis (Van den Eijnde SM, et al., Cell death Diff. 1997;4:311-316). Loss of CMLA occurs early in the apoptotic cascade, immediately following the point of cell commitment to the death process (Van-Engeland M, et al., Cytometry 1998;31:1-9; Martin SJ, et al., J. Exp. Med. 1995;182:1545-1556). It has also been shown that the loss of CMLA is an important factor in the recognition and removal of apoptotic cells by macrophages (Balasubramanian K, et al., J. Biol. Chem. 1997;272:31113-31117). A strong correlation has recently been drawn between the loss of CMLA and the potent procoagulant activity of apoptotic cells (Bombeli T, et al., Blood 1997; 89:2429-2442; Flynn PD, et al., Blood 1997;89:4378-4384). The latter activity in apoptotic endothelial cells, such as those recently recognized in atherosclerotic plaques (Kockx MM, et al., Circulation 1998;97:2307-2315, Mallat Z, et al., Circulation 1997;96:424-428), probably plays an important role in the pathogenesis of thrombotic vascular disorders.

The diagnosis of the loss of CMLA may therefore serve as an important tool for the detection of cell death, specifically by apoptosis. A method for the detection of cell death and for the removal of the dead cells and/or cell-derived particles may have many applications, particularly for the filtration of biological fluids, in order to remove apoptotic cells, cell-derived particles, and other potentially procoagulant particles.

The potential utility of a detector of CMLA loss both as a diagnostic tool and as a therapeutic apparatus has recently been exemplified by the use of annexin-V for the diagnosis and detection of CMLA loss. Annexin V is a member of the annexin family of proteins, sharing potent, Ca^{2+}-dependent binding to anionic phospholipid membranes (Swairjo MA, et al., Nature Struc. Biol. 1995;968-974). Annexin V is a 320 amino acid protein, with a molecular mass of 35,935 daltons (Huber R, et al., EMBO J. 1990;9:3867-3874). Though the physiological role of annexin-V has not been fully elucidated, it has been suggested to be involved in anticoagulation, anti-inflammation and cellular signaling (Romisch J, et al., Thromb. Res. 1990;60:355-366; Bastian BC, J. Invest. Dermatol. 1993; 101:359-363; Kaneko N, et al., J. Mol. Biol.

1997;274:16-20). The impressive affinity of annexin V to anionic phospholipid membranes (Kd of about 10^{-9} -10^{-11}M, [Hofmann A, et al., Biochim. Biophys. Acta, 1997;254-264]) has been extensively utilized for both the diagnosis of CMLA loss and modulation of disorders associated with this phenomenon. Fluorescein isothiocyanate (FITC)-labeled annexin V has been widely used for the detection of apoptosis in various tissue culture models (Koopman G, et al., Blood 1994;84:1415-1420; Rimon G, et al., J Neurosci Res 1997; 48:563-570;Van-Engeland M, et al., Cytometry 1998;31:1-8). Preliminary successful studies were also performed with systemic intracardial injection of biotinylated annexin V to viable mouse embryos, for the detection of developmentally-associated apoptosis (Van den Eijnde SM, et al., Cell Death Diff. 1997;4:311-316). Systemic administration of ^{99m}Tc-annexin V was also used to detect and image cell death in several models in vivo, e.g. fulminant hepatitis in mice, acute rejection of transplanted cardiac allograft in rats and monitoring of response of lymphoma to cyclophosphamide treatment in mice (Blankenberg FG. et. al. Proc. Natl. Acad. Sci. USA, 95:6349-6354, 1998). ^{125}I-labeled annexin V was also used for in vivo detection of thrombosis in an animal model (Stratton JR, et al., Circulation 1995;92:3113-3121). Inhibition of arterial thrombosis was effectively achieved by intravenous administration of annexin V in a carotid artery injury model (Thiagarajan P & Benedict CR, Circulation 1997;96:2339-2347). Annexin V is also known as diagnostic agent (U.S. Patent No. 5,627,036).

However, the use of annexin V as a drug or as a diagnostic probe is rendered problematic by several characteristics of this protein. Annexin V is a protein of considerable size, a factor which may substantially limit its volume of distribution in the body. Moreover, it is active as a potent membrane-binding protein only if allowed to form a highly organized multimer on the membrane surface (Concha NO, et al., FEBS Lett 1992;314:159-162.; Voges D, et al., J. Mol. Biol. 1994;238:199-213, Andree HAM, et a., J. Biol. Chem 1992;267:17907-17912). Thus, systemic administration of annexin V as a drug is expected to be associated with rapid degradation and loss of the function of the administered protein. Indeed, a very rapid clearance (90% within 5 minutes) was observed in rabbits following intravenous injection of annexin V (Thiagarajan P & Benedict CR, Circulation 1997;96:2339-2347). In addition, the administration of annexin V may induce an untoward immunological response. Importantly, anti-annexin V antibodies have been recently implicated in the pathogenesis of anti-phospholipid antibody syndrome and associated thrombotic events (Nakamura N, et al., Am. J. Hematol. 1995; 49:347-348; Kaburaki J & Ikeda Y, Rinsho Ketsueki 1995;36:320-324, Rand JH , et al., N. Engl. J. Med. 1997;337:154-160). Furthermore, as a protein, annexin V may be expected to suffer from

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potential instability and susceptibility to degradation.

In order to overcome these problems with the annexin V protein, clearly compounds are required which share the desirable affinity for anionic phospholipids of annexin V, without the drawbacks associated with that protein. Israeli Patent Application No. 125908, filed August 24, 1998, which is incorporated by reference as if fully set forth herein, teaches a novel family of compounds, which are also characterized by potent binding to cell membranes upon surface exposure of anionic phospholipids, i.e., membranes characterized by CMLA loss. The members of this family, which are referred to hereinafter as the "NST300 compounds", are of the general formula (I):

$$X_1\ [(X_3)_a / (X_4)_b]$$

wherein:

a and b are integers independently selected from 1 to 8.

X_1 includes a saturated or unsaturated fatty acid residue including 6 - 20 carbon atoms; or a cysteine residue bound through a thioether bond to a prenyl group including 5 - 20 carbon atoms. The residue is linked to the adjacent component of the compound through an amide bond.

X_3 includes 1-6 amino acid residues, of which 1-6 amino acid residues are positively charged, the other amino acid residues are polar uncharged amino acids.

X_4 includes 1-6 amino acid residues, of which 1-2 residues are aromatic amino acid residues, the other amino acid residues are polar uncharged amino acid residues and/or hydrophobic aliphatic amino acid residues.

The X_3 and X_4 groups according to Israeli Patent Application No. 125908 can be placed at various locations in the compound.

For the sake of clarity it should be indicated that the term "prenyl" as used herein stands also for the term "isoprenyl" (see Stedman's Medical Dictionary, Baltimore, USA, William and Wilkins, eds., 1990:565, 1253).

X_1 according to Israeli Patent Application No. 125908 serves as main anchoring domain A. X_3 serves as an anionic phospholipid binding determinant; and X_4 serves as accessory anchoring domain.

X_1 according to Israeli Patent Application No. 125908 is advantageously a residue of a saturated fatty acid of formula $CH_3(CH_2)_nCO_2H$, in which n stands for an integer of 8 - 18, preferably selected from the group consisting of myristic acid and palmitic acid; or X_1 is advantageously a cysteine residue bound through a thioether bond to a prenyl of 5 - 15 carbon

atoms, preferably farnesyl cysteine.

The positively charged amino acid residues of X_3 according to Israeli Patent Application No. 125908 are advantageously selected among lysine, arginine, histidine or any amino acid residue which includes a positively charged group, e.g., primary amine, secondary amine, guanidine, covalently bound to the α-carbon atom or to the α-amine on the peptide backbone by a spacer which includes alkane or alkene of 1 - 4 carbon atoms; and combinations thereof.

The acids are preferably selected among lysine and arginine and combinations thereof. The polar uncharged amino acids of X_3 are preferably selected among serine, threonine, asparagine and glutamine and combinations thereof.

The aromatic amino acid residues of X_4 are preferably selected from the group consisting of phenylalanine and tryptophan residues and combinations thereof. The polar uncharged amino acid residues are preferably selected among serine, asparagine and glutamine residues and combinations thereof; and the hydrophobic aliphatic amino acid residues are preferably selected among leucine, alanine and glycine residues and combinations thereof.

The compound according to Israeli Patent Application No. 125908 potentially further includes additional groups: X_2, X_5 and X_6, in which case it can be described by general formula Ia:

$$X_1 - X_2 - ((X_3)_a / (X_4)_b / (X_5)_c) - X_6 \quad \text{(Ia)}$$

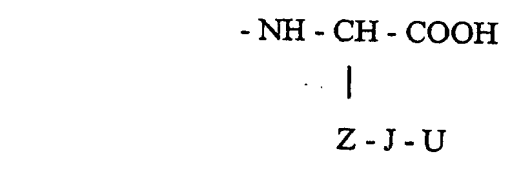
wherein:

c is an integer between 0 and 10.

X_1, X_3 and X_4 are as described above.

X_2 is selected among 0 - 3 glycine residues and 0 - 2 ?-amino alanine molecules;

X_5 is a compound of general formula II:



wherein Z represents a spacer group selected from a saturated alkane and a non-saturated alkene containing 1 - 5 carbon atoms; J represents a functional group selected from amines, thiols, alcohols, carboxylic acids and esters, aldehydes and alkyl halides; and U is an anchoring (binding) group or X_1 group (as hereinbefore defined).

X_6 is either zero or an X_1 group (as hereinbefore defined);

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As further described in Israeli Patent Application No. 125908, within the subunit $[(X_3)_a/(X_4)_b/(X_5)_c]$, the groups X_3, X_4 and X_5 can be located at various suitable places.

X_2 according to Israeli Patent Application No. 125908 serves as linker A, between X_1 and the sub-unit $[(X_3)_a / (X_4)_b]$ or between X_1 and the sub-unit $[(X_3)_a / (X_4)_b / (X_5)_c]$.

X_5 according to Israeli Patent Application No. 125908 serves as linker B between the subunit $(X_3)_a / (X_4)_b$ and X_6 or between the subunit $(X_3)_a / (X_4)_b / (X_5)_c$ and X_6.

X_6 serves as a main anchoring domain B.

X_5 is advantageously a lysine residue substituted at the ε-amino group by an anchoring group such as, but not limited to, biotin.

In case that X_6 stands for a cysteine residue bound through a thioether bond to a prenyl group the cysteine carboxyl group can be either free or methylated.

Any of the above amino acid residues may be the L isomer, the D isomer or the DL racemate.

The amino acid residues may also be residues of suitable synthetic amino acid residues.

An exemplary presently preferred sequence of the compounds of the general formulae described above is:

myristate-GGGKKKKKRFSFKKSFKLSGFSFKKNKKKU (SEQ ID NO:1), in which G=glycine, K=lysine, R=arginine, F=phenylalanine, S=serine, L=leucine, N=asparagine and U as hereinbefore defined. A preferred compound of this sequence is:

myristate-GGGKKKKKRFSFKKSFKLSGFSFKKNKKK(biotin) (SEQ ID NO:1), which is referred to hereinafter as the "NST301 compound".

Another sequence of the compounds of the general formulae described above is:

myristate-KKKKKRFSFKKSFKLSGFSFKKNKKKU, (SEQ ID NO:2), wherein K, R, F, S, L, G, N and U have the same meaning as above.

A preferred compound of the sequence is:

myristate-KKKKKRFSFKKSFKLSGFSFKKNKKK(biotin) (SEQ ID NO:2). This compound is referred to herein as the "NST302 compound".

Israeli Patent Application No. 125908 also discloses a process for the preparation of the NST300 compounds of general formula I by the following steps.

For the preparation of sub unit $[(X_3)_a / (X_4)_b]$ an α-amine protected, C terminal amino acid of the sequence is loaded on a solid support, the α-amine protecting group is removed, and the peptide sequence is sequentially prepared.

For coupling of X_1, the α-amino protecting group is removed from the N-terminal

amino acid residue, and X_1 is then introduced into the peptide-resin conjugate under the same conditions as above.

Finally, the peptide is cleaved from the solid support, purified and characterized.

NST300 compounds of general formula Ia comprising sub-unit $[(X_3)_a / (X_4)_b / (X_5)_c)]$ are prepared as described above.

For the preparation of X_5 and its coupling to an anchoring group or to X_6 an orthogonally protected amino acid is loaded on a solid support; the protecting group on the ω-functional group is selectively removed; X_6 or the anchoring group of X_5 is introduced into the amino acid-resin in the presence of an appropriate coupling reagent or by using a pre-activation method.

The coupling agent may be HBTU/HOBT and the pre-activation method may be the formation of an ester, azide or an anhydride.

The integration of X_5 (either coupled to an anchoring group or coupled to X_6) into the peptide sequence can be effected as described above.

The characterization is preferably performed by high performance liquid chromatography - mass spectra (HPLC-MS).

The above synthesis and the pre-activation can easily be performed by one of ordinary skill in the art of solid phase peptide synthesis (Atherton E, Sheppard RC, Solid phase peptide synthesis; a practical approach, IRL Press, 1989; Bodanszky M, Peptide Chemistry, Springer Verlag, 1988).

As disclosed in Israeli Patent Application No. 125908 and as exemplified in the Examples section hereinunder, the compounds according to formulae I and Ia are effective in binding to cells upon CMLA loss, specifically in binding to cells undergoing apoptosis.

The present invention takes specific advantage of these synthetic compounds, another class of cyclized synthetic compounds referred to herein as the "NST500 group", as well as of natural and recombinant proteins of the annexin family and portions and analogs thereof to provide affinity filters incorporating same, which filters can be used to capture and remove particles characterized by surface exposure of anionic phospholipids from blood or blood-derived products prior to transfusion thereof or during extracorporeal circulation, as well as for the filtration of other biological fluids.

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SUMMARY OF THE INVENTION

It is one object of the present invention to provide apparatus and method for capture and removal from biological fluids such as the blood or from blood-derived products of particles characterized by surface exposure of anionic phospholipids, therefore hereby designated anionic phospholipid-exposing particles (APEP). These particles are, for example, one or more of the following: (i) damaged blood and endothelial cells; (ii) senescent blood cells; (iii) activated platelets; (iv) microparticles generated from platelets or from other cell types; (v) cells undergoing apoptosis or apoptotic bodies. Due to the surface exposure of the anionic phospholipids, these particles are potentially hazardous, as they can exert procoagulant effects, and/or act as microemboli, occluding and/or initiating thrombosis in small blood vessels.

It is another object of the present invention to provide an apparatus and method for directing biological fluids such as blood or a blood-derived product through an affinity filter device. The filter device includes a compound, capable of selective and high-affinity binding to anionic phospholipid membranes, hereby designated anionic phospholipid-binding compound (APBC). APBC is stably (e.g., covalently) attached to a synthetic polymeric matrix either directly or through a spacer. Upon passing through the filter, APEP in the blood or in the blood-derived product come in contact with and bind to the APBC, such that the APBC thus captures and clears the APEP from the circulation.

It is yet another object of the present invention to provide such a filter device and method, in which the APBC is an NST300 compound. NST300 compounds are a novel group of compounds, described in Israeli Patent Application No. 125908, filed August 24, 1998, capable of high-affinity binding to anionic phospholipid membranes. Acting through this binding, these compounds have been shown to be potent detectors of apoptotic cells, and have also been shown to exert anticoagulant effects. In addition, within the context of the filter device and the method of use thereof, the NST300 compounds have been shown to be able to capture apoptotic cells and cell-derived particles which display anionic phospholipid membranes, and hence to be able to filter such particles from biological fluids, such as blood for transfusion, for example.

It is an alternative object of the present invention to provide such a filter device and method, in which the APBC is an NST500 compound. NST500 compounds are a second novel group of compounds, described in Israeli Patent Application No. 131266, filed on August 5, 1999, which are capable of high-affinity binding to anionic phospholipid membranes. Although such NST500 compounds have certain functional capabilities with regard to filtration

of biological fluids which are similar to those capabilities of the NST300 compounds, NST500 compounds are structurally very different, and feature comparable or improved binding characteristics. Thus, the NST500 compounds represent an improved group of compounds, in terms of their higher overall activity, stability and ability to provide high-affinity binding to anionic phospholipid membranes for the filtration of biological fluids.

It is yet another alternative object of the present invention to provide such a filter device and method, in which the APBC is an annexin or a portion thereof, for example, annexin V. Annexin V is 36 kDa protein, that binds PS-containing membranes, in a calcium-dependent manner, with a very high affinity (Voges D, et al., J Mol Biol 1994;239:199-213). Annexin V is widely used as a probe for detection of cells undergoing apoptosis *in vitro* (Koopman G, et al., Blood 1994;84:1415-1420), and also as a detector of anionic-phospholipid- mediated procoagulant states (Stratton JR, et al., Circulation 1995;92:3113-3121). Annexin V has also been used in preliminary *in vivo* studies to inhibit arterial thrombosis (Thiagarajan P & Benedict CR, Circulation 1997;96:2339-2347).

It is still another object of the present invention to use the above-mentioned filter to improve cardiopulmonary bypass procedures, i.e., procedures requiring extracorporeal circulation, by reducing the risk for thromboembolic events during the procedure, by removal of potentially harmful particles from the blood.

It is an additional object of the present invention to reduce the risk for microemboli during cardiopulmonary bypass, by placing the above-described affinity filter on the line for passing blood from the extracorporeal circulation machine to the patient, thus filtering out the microemboli.

It is yet an additional object of the present invention to improve quality of transfused blood and blood-derived products, by removing defective or senescent cells or by removing microparticles generated during storage or due to storage conditions.

It is still an object of the present invention to eliminate defective or senescent cells in blood or blood-derived products by passing the blood or blood-derived product through the affinity filter, optionally placed on the intravenous line, before administration of the blood or the blood-derived product to the patient.

Thus, according to one aspect of the present invention there is provided an affinity filter effective in capturing and thereby removing particles characterized by surface exposure of anionic phospholipids present in blood or blood-derived products, the affinity filter comprising a body having an inlet and an outlet, the body including a solid support and an anionic-

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phospholipid binding compound (APBC) linked, either directly, or indirectly via a spacer, to the solid support, the anionic-phospholipid binding compound being for specifically binding the particles characterized by surface exposure of anionic phospholipids and thereby removing the particles from the blood or blood-derived products.

According to another aspect of the present invention there is provided a method of capturing and thereby removing particles characterized by surface exposure of anionic phospholipids present in blood or blood-derived products, the method comprising the step of directing the blood through an affinity filter including a body having an inlet and an outlet, the body including a solid support and an anionic-phospholipid binding compound linked to the solid support, the anionic-phospholipid binding compound being for specifically binding the particles characterized by surface exposure of anionic phospholipids and thereby removing the particles from the blood or blood-derived products.

According to further features in preferred embodiments of the invention described below, the body and the solid support are selected so as to allow the blood or blood-derived products to flow through the body while substantially maintaining the integrity of intact blood cells flowing therethrough.

According to still further features in the described preferred embodiments the solid support is selected from the group consisting of an organic solid support and inorganic solid support.

According to still further features in the described preferred embodiments the solid support is formed of a structure selected from the group consisting of microparticulates, microfibers, beads and microcapillaries. The beads are more preferably selected from the group consisting of resin beads and magnetic beads. The magnetic beads are most preferably selected from the group consisting of microparticles and nanoparticles.

According to still further features in the described preferred embodiments the anionic-phospholipid binding compound is an annexin or a portion thereof effective in binding the particles characterized by surface exposure of anionic phospholipids and thereby removing the particles from the blood or blood-derived products.

According to still further features in the described preferred embodiments the annexin is selected from the group consisting of annexin I, annexin II, annexin III, annexin IV, annexin VI, annexin VII, annexin VIII, annexin XI and annexin XIII.

According to still further features in the described preferred embodiments the annexin is annexin V.

According to still further features in the described preferred embodiments the annexin is recombinant.

According to still further features in the described preferred embodiments the annexin is natural.

According to still further features in the described preferred embodiments wherein the anionic-phospholipid binding compound is of a general formula (hereinafter a compound belonging to the group designated as "NST300"):

$$X_1\ [(X_3)_a / (X_4)_b]$$

wherein:

a and *b* are each independently an integer between 1 and 8;

X_1 is selected from the group consisting of a saturated fatty acid residue of 6 - 20 carbon atoms, an unsaturated fatty acid residue of 6 - 20 carbon atoms, a polyunsaturated fatty acid residue of 6 - 20 carbon atoms, and a cysteine residue bound through a thioether bond to a prenyl group of 5 - 20 carbon atoms, the residue being linked to an adjacent component of the compound through an amide bond;

X_3 comprises 1-6 amino acid residues, of which at least one amino acid residue is positively charged, wherein the remaining amino acid residues being polar uncharged amino acid residues; and

X_4 comprises 1-6 amino acid residues, of which 1-2 are aromatic amino acid residues, the remaining amino acid residues are selected from the group consisting of polar uncharged amino acid residues and hydrophobic amino acid residues;

whereas, groups X_3 and X_4 present are arranged in one of all possible linear arrangements with respect to one another.

According to still further features in the described preferred embodiments X_1 is a residue of a saturated fatty acid of a formula $CH_3(CH_2)_nCO_2H$,in which *n* is an integer between 8 - 18, or X_1 is a cysteine residue bound through a thioester bond to a prenyl of 5 - 15 carbon atoms.

According to still further features in the described preferred embodiments X_1 is selected from the group consisting of myristic acid, palmitic acid and farnesyl cysteine.

According to still further features in the described preferred embodiments the positively charged amino acid residues of X_3 are selected from the group consisting of lysine residue, arginine residue, histidine residue and an amino acid which includes a positively charged group covalently bound to an α-carbon atom or to an α-amine on a peptide backbone by a spacer

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which is a carbon chain of 1 - 5 carbon atoms, substituted or unsubstituted, saturated or unsaturated, preferably selected from the group consisting of alkane or alkene, and combinations thereof.

According to still further features in the described preferred embodiments the positively charged amino acid residues in X_3 are selected from the group consisting of lysine and arginine residues and combinations thereof.

According to still further features in the described preferred embodiments the polar uncharged amino acid residues of X_3 are selected from the group consisting of serine, threonine, asparagine and glutamine residues and combinations thereof.

According to still further features in the described preferred embodiments the aromatic amino acid residues of X_4 are selected from the group consisting of phenylalanine and tryptophan residues and combinations thereof.

According to still further features in the described preferred embodiments the polar uncharged amino acid residues of X_4 are selected from the group consisting of serine, asparagine and glutamine residues and combinations thereof.

According to still further features in the described preferred embodiments the hydrophobic aliphatic amino acid residues of X_4 are selected from the group consisting of leucine, alanine and glycine residues and combinations thereof.

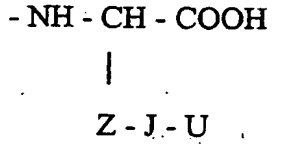
According to still further features in the described preferred embodiments the anionic-phospholipid binding compound includes additional groups X_2, X_5 and X_6 and which has a general formula Ia:

$$X_1\ X_2\ [(X_3)_a / (X_4)_b /(X_5)_c]\ X_6$$

wherein:

X_2 includes 0 - 3 glycine residues and 0 - 2 β-amino alanine molecules;

X_5 is selected from the group consisting of a linker βA-βA-P-βA-βA and a compound of a general formula II:



wherein:

Z is a spacer group containing a carbon chain of 1 - 5 carbon atoms, saturated or unsaturated, substituted or unsubstituted, preferably alkane or alkene;

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J is a functional group selected from the group consisting of amines, thiols, alcohols, carboxylic acids, esters, aldehydes and alkyl halides;

U is an anchoring group or the X_1 group;

c is an integer from 0 - 10; and

X_6 is zero or the X_1;

whereas, within subunit $[(X_3)_a/(X_4)_b/(X_5)_c]$ the groups X_3, X_4 and X_5 are arranged in one of all possible linear arrangements with respect to one another.

According to still further features in the described preferred embodiments X_5 is a lysine residue being substituted at an ε-amino group thereof by an anchoring group.

According to still further features in the described preferred embodiments the anionic-phospholipid binding compound is:

myristate-GGGKKKKKRFSFKKSFKLSGFSFKKNKKKU.

According to still further features in the described preferred embodiments the anionic-phospholipid binding compound is:

myristate-GGGKKKKKRFSFKKSFKLSGFSFKKNKKK(biotin). Alternatively and more preferably, the anionic-phospholipid binding compound is:

myristate-GGGKKKKKRFSFKKSFKLSGFSFKKNKKK-βA-βA-P-βA-βA.

According to still further features in the described preferred embodiments the anionic-phospholipid binding compound is:

myristate-KKKKKRFSFKKSFKLSGFSFKKNKKKU.

According to still further features in the described preferred embodiments, the anionic-phospholipid binding compound is:

myristate-KKKKKRFSFKKSFKLSGFSFKKNKKK(biotin). Alternatively and more preferably, the anionic-phospholipid binding compound is:

myristate-KKKKKRFSFKKSFKLSGFSFKKNKKK-βA-βA-P-βA-βA.

According to yet another preferred embodiment of the present invention, there is provided a group of compounds, generally designated as "NST500" compounds. These compounds may be generally described as also including a cyclized form of the compounds of general formula I or Ia, such that the cyclized compound features at least a fatty acid moiety and a plurality of amino acid residues. Preferably, the NST500 compounds are compounds of a general formula III:

X_1-X_2-$\{(X_3)_a/X_4/X_5\}$-X_6

wherein:

X_1 stands for a saturated or unsaturated fatty acid residue comprising 6 - 20 carbon atoms; or a cysteine residue bound through a thioether bond to a prenyl group comprising 5 - 20 carbon atoms; the residue being linked to the adjacent component of the compound through an amide bond;

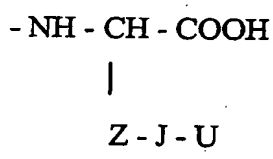
X_2 is either 0 or includes at least one unit of a general formula (IV) A^*2^n, in which A stands for a branching unit and *n* stands for 0 - 4;

X_3 comprises 1-6 amino acids, of which 1-6 are positively charged and 0-2 are negatively charged, any remaining amino acid residues being polar uncharged amino acids;

X_4 comprises 1-6 amino acids, of which 1-2 are aromatic amino acids, any remaining amino acids being selected among polar uncharged amino acids and hydrophobic aliphatic amino acids;

X_5 comprises 1-6 amino acids, of which 1-6 are positively charged and 0-2 are negatively charged, any remaining amino acid residues being polar uncharged amino acids, wherein the amino acids form a cyclic structure;

X_6 is selected from the group consisting of a linker, such as βA-βA-P-βA-βA for example, or a compound of the general formula II



wherein Z stands for a spacer group containing a chain of 1 - 5 carbon atoms, saturated or unsaturated, substituted or unsubstituted, preferably alkane or alkene; J represents a functional group selected among amines, thiols, alcohols, carboxylic acids and esters, aldehydes and alkyl halides; U is either 0 or a labeling moiety;

wherein:

a is an integer of 1 - 3; and

the groups X_3, X_4 and X_5 are each placed at an individually selected location in the compound, thereby selecting one of any possible linear combination of these groups;

as well as functional equivalents thereof and/or compounds having the same biological activity thereto.

For the sake of clarity it should be indicated that the term "prenyl" herein stands also for the term "isoprenyl" (see Stedman's Medical Dictionary, Baltimore, USA, William and Wilkins, eds., 1990:565, 1253).

The function of each component of the general formula III is as follows:

X_1 serves as main anchoring domain;

X_2, if present, serves as branching domain;

X_3 serves as anionic phospholipid binding determinant;

X_4 serves as accessory anchoring domain;

X_5 serves as anionic phospholipid binding domain bearing a cyclic conformation; and

X_6 serves as a labeling domain.

X_1 is advantageously a residue of a saturated fatty acid of formula $CH_3(CH_2)_nCO_2H$, in which *n* stands for an integer of 8 - 18 preferably selected among myristic acid and palmitic acid; or X_1 is advantageously a cysteine residue bound through a thioether bond to a prenyl of 5 - 15 carbon atoms, preferably farnesyl cysteine.

Branching unit A of X_2 is advantageously selected among a di-carboxylic or a poly-carboxylic acid.

The positively charged amino acids of X_3 and X_5 are advantageously selected among lysine, arginine, histidine or any amino acid which is comprised of a positively charged group, e.g. primary amine, secondary amine, guanidine, covalently bound to an α-carbon atom or to an α-amine on the peptide backbone by a spacer comprised of an alkene of 1 - 5 carbon atoms; and combinations thereof. The acids are preferably selected among lysine and arginine and combinations thereof. The negatively charged amino acids of X_3 and X_5 are preferably selected among glutamate and aspartate. The polar uncharged amino acids of X_3 and X_5 are preferably selected among serine, threonine, asparagine and glutamine and combinations thereof.

The aromatic amino acids of X_4 are preferably selected among phenylalanine and tryptophan and combinations thereof; the polar uncharged amino acids are preferably selected among serine, asparagine and glutamine and combinations thereof; and the hydrophobic aliphatic amino acids are preferably selected among leucine, valine, alanine and glycine and combinations thereof.

The cyclization in X_5 may be performed via an intra-molecular di-sulfide bridge, by an amide bond or via a coordination bond to a metal, preferably to ^{99}Tc, that may serve also as a marker.

U as a labeling moiety for detection of specific binding is advantageously selected among biotin and a group containing a substituent selected among a fluorescein, a radioisotope and a paramagnetic contrast agent; the fluorescein may be, for example, fluorescein isothiocyanate; the radioisotope may be selected among technetium, lead, mercury, thallium and indium; and the paramagnetic contrast agent may be any paramagnetic metal ion chelate, e.g. gadolinium-diethyl-enetriaminepentaacetic acid (Gd-DTPA).

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X_6 is advantageously a lysine residue substituted at the ε-amino group by a labeling group U as above defined.

If X_6 represents a cysteine residue bound through a thioether bond to a prenyl group, the cysteine carboxyl group can be either free or methylated.

Any of the above amino acids may be the L-, the D- or the DL isomer or the racemate.

The amino acid residues may also be residues of suitable synthetic amino acids.

One exemplary but preferred sequence of the compounds of general formulae III is:

Myristate - KVSFFCKNKEKKC - KU, in which

K=lysine, V=Valine, S=serine, F=phenylalanine, C=Cysteine, N=asparagine, E=Glutamic acid and U are as hereinbefore defined.

A particularly preferred but exemplary compound of the sequence is:

Myristate - KVSFFCKNKEKKC - K in which the two cysteine residues are linked via a disulfide bond, to form a cyclic structure (this compound is herein called "NST513"). Optionally, a biotin may be attached to the compound as an example of the U moiety. Alternatively and preferably, NST513-B is prepared, which has the sequence Myristate - KVSFFCKNKEKKC – K-βA-βA-P-βA-βA, such that the compound is attached to the linker βA-βA-P-βA-βA.

A still further preferred but exemplary compound of the sequence is:

Myristate - KKVFSFCKNKEKKC - K wherein the two cysteine residues are linked together to form a cyclic structure (this compound is herein called "NST516"). Again, optionally, a biotin may be attached to the compound as an example of the U moiety, or alternatively the linker βA-βA-P-βA-βA may be attached.

According to yet other features of the described preferred embodiments for the affinity filter of the present invention, the solid support for the affinity filter includes a plurality of beads to which the anionic-phospholipid binding compound is attached. More preferably, the beads are resin containing beads. Alternatively and more preferably, the beads are magnetic beads which are most preferably microparticles or nanoparticles. Although the affinity filter of the present invention is described with regard to certain specific anionic-phospholipid binding compounds being attached to the beads, it is understood that substantially any such compound could be used with the beads in the affinity filter of the present invention, including but not limited to, the NST300 group of compounds; the NST500 group of compounds; and annexin or a portion thereof, for example, annexin V.

According to still further features in the described preferred embodiments the affinity filter forms a portion (e.g., permanent, replaceable, add-on portion) of an extracorporeal

circulation apparatus.

According to still further features in the described preferred embodiments the affinity filter forms a portion (e.g., permanent, replaceable, add-on portion) of a blood transfusion apparatus.

According to still further features in the described preferred embodiments the affinity filter forms a portion (e.g., permanent, replaceable, add-on portion) of a hemodialysis apparatus.

According to still further features in the described preferred embodiments the affinity filter forms a portion (e.g., permanent, replaceable, add-on portion) of a plasmapheresis apparatus.

According to still further features in the described preferred embodiments the blood is of a patient selected from the group consisting of a patient undergoing a surgery, a patient undergoing hemodialysis, a patient undergoing a plasmapheresis, a diabetic patient, a thalassemic patient, and a patient having systemic lupus erythematosus.

Hereinafter, the term "biological fluid" includes, but is not limited to, blood, blood-derived products, urine, cerebrospinal fluid (CSF), semen, mucous, and substantially any other fluid found in an organism, of which a particularly preferred example is blood and blood-derived products.

The present invention successfully addresses the shortcomings of the presently known configurations by providing an affinity filter and a method of using same, effective in removal of microparticles, such as microemboli, from blood and blood-derived products prior to their transfusion and/or microemboli generated during extracorporeal circulation.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention herein described, by way of example only, with reference to the accompanying drawings, wherein:

FIGS. 1A-C show exemplary structures of the NST compounds used in the affinity filter according to the present invention, wherein Figure 1A shows an example of the main structural domains of the NST 300 compounds (general formula Ia), Figure 1B shows a detailed structure of the NST301 compound, and Figure 1C shows a detailed structure of the NST302 compound (PS = phosphatidylserine, the main anionic phospholipid exposed on cell surface upon CMLA loss);

FIGS. 2A-G demonstrate binding of the NST301 compound to single apoptotic cells

using a morphological study, in which Figures 2A-C show cultured HeLa cells undergoing dopamine (DA)-induced apoptosis (HeLa cells grown on slides were induced to undergo apoptosis by 500 μM of DA for 18 hours, after which apoptotic cells were identified by Hoechst 33342 staining; Figure 2A - control, non treated cells, 2B - DA-induced apoptosis in HeLa cells. Some of the apoptotic cells are indicated by arrows. Figure 2C - DA-induced apoptotic HeLa cells after staining with Hoechst 33342. The same field as in Figure 2B is presented, and the arrows point to the same apoptotic cells as in 2B. 50 % of the cells treated according to the above protocol were identified as apoptotic cells. Magnification is times 270. Figures 2D-E show detection of apoptotic cells by the NST301 compound. Control HeLa cells (2D) and dopamine (DA)-treated cells (2E) were stained by 750 nM of the biotinylated NST301 compound and visualized by fluorescent microscopy following the application of streptavidin reagent labeled with FITC. Although many healthy cells were present in this field (2D), only few of them were stained with NST301, and very faintly. Apoptotic cells were stained by NST301, and typical cells are presented and marked by arrows in Figure 2E. Magnification is times 460. Figures 2F-G show staining by the NST 301 compound of cells undergoing various stages of apoptosis. Early apoptotic (light, peripheral staining) versus late apoptotic (intense labeling). Magnification is times 1200);

FIGS. 3A-C demonstrate detection of apoptotic cells by NST compounds using flow cytometric (FACS) analysis (Figure 3A shows results obtained with the NST301 compound. Three different types of cell populations (control, early and advanced apoptotic cells) were subjected to 3 different staining protocols: (i) PI and streptavidin reagent labeled with FITC (control, no NST300 compound); (ii) double staining with PI and NST301 followed by application of streptavidin reagent labeled with FITC; and (iii) double staining with PI and NST301-C (control compound, GGGKKKKKRFSFKKSFKLSGFSFKKNKKK(Biotin)-OH (SEQ ID NO:3)) followed by application of streptavidin reagent labeled with FITC. For each of the treatments, the percentage of cells that were stained with FITC (indicative of binding of NST301 compound) and cells that were stained with PI, (indicative of loss of plasma membrane integrity, typical of advanced apoptotic stages) were determined. NST300 compounds used for staining were at a concentration of 750 nM. As shown, NST301, but not the control compound NST301-C, was a potent marker of the apoptotic cells. Figure 3B shows binding of different NST300 compounds to apoptotic cells. Control non-treated cells and early apoptotic cells were stained with 750 nM of different NST300 compounds. Binding intensity was defined as the ratio between FITC mean value of apoptotic cells to that of control cells. The NST301

compound showed a 4 fold increase in FITC intensity as compared to control. The NST302 showed a 9 fold increase in binding intensity. Figure 3C shows detection of early apoptotic cell populations by NST300 compounds. The ratio between the percentage of HeLa cells stained with FITC to the percentage of cells stained with PI was defined as an indicator of early apoptotic cell populations. These cells, in the beginning of the death process, are characterized by loss of CMLA, while still retaining plasma membrane integrity. Before staining with FITC and PI, early and advanced apoptotic cells were exposed to either one of the followings: no treatment, treatment with NST301, and treatment with NST301-C. As shown, NST301 compound was a potent detector of the early apoptotic cells);

FIG. 4 demonstrates the anticoagulant effect of the NST300 compounds using the Russell viper venom (RVV) test (Clotting time ratio was calculated as the ratio of clotting time measured with normal plasma, pretreated with NST300 compounds, versus clotting time measured with non-treated normal plasma. The NST300 compounds manifested potent anticoagulant effect, with NST302 exerting the most powerful effect. The graph represents three independent experiments);

FIG. 5 demonstrates that NST300 compounds (500 nM) potently correct the procoagulant effect of apoptotic cells (A modified APTT coagulation test was used to determine the procoagulant activity of apoptotic cells. Samples of control non-apoptotic cells and apoptotic cells were used with or without preincubation with NST300 compounds. Reactions were performed in duplicates, and the graph represents three independent experiments. NST301 and NST302 were potent in fully reversing the marked procoagulant effect of the apoptotic cells. By contrast, only a very modest effect was exerted by the control compound NST 301-C. Reference is being made to the descriptions in Examples 2 to 4);

FIG. 6 shows an affinity filter according to the present invention;

FIG. 7 is a photograph of fluorescence microscopy after incubation of apoptotic HeLa cells with either control TentaGel beads or beads coated with NST302, demonstrating that NST302-coated beads bind specifically to the apoptotic cells;

FIG. 8 is a photograph of confocal microscopy after incubation of apoptotic HeLa cells with either control TentaGel beads or beads coated with NST302, demonstrating that NST302-coated beads bind specifically to the apoptotic cells;

FIG. 9 is a graph showing that treatment of apoptotic HeLa cells with NST302-coated beads increases coagulation time; and

FIG. 10 shows a graph of the percentage of FITC positive cells after treatment with

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control TentaGel beads, and 7.5 mg or 25 mg NST513-coated beads, showing that NST513-coated beads selectively bind activated platelets.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of apparatus and method for capturing and thereby removing particles characterized by surface exposure of anionic phospholipids from biological fluids, preferably including blood or blood-derived products. Specifically, the present invention can be used to lower the level of, or substantially eliminate microparticles, such as microemboli, present in either transfused blood or blood-derived products or in blood undergoing extracorporeal circulation. Most specifically, the present invention can be used to lower the risk of microemboli potentially causing organ damage and dysfunction following extracorporeal circulation and/or blood transfusion. The present invention also features novel anionic phospholipid-binding compounds, both linear compounds (NST300 group) and cyclized compounds (NST500 group), which are demonstrated herein to have particular utility for the filtration of biological fluids.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Referring now to the drawings, Figure 6 illustrates an affinity filter according to one aspect of the present invention, which is referred to hereinunder as an affinity filter **10**.

Affinity filter **10** according to the present invention is effective in capturing and thereby removing particles characterized by surface exposure of anionic phospholipids present in biological fluids such as blood or blood-derived products. Affinity filter **10** includes a body **12** formed with an inlet **14** and an outlet **16**. Body **12** contains a solid support **18** and an anionic-phospholipid binding compound, linked, either directly, or indirectly via a spacer, such as, but not limited to, alkane or alkene, to solid support **18**. The anionic-phospholipid binding compound serves for specifically binding the particles characterized by surface exposure of anionic phospholipids and thereby removing the particles from the biological fluid such as the

blood or blood-derived products. A net structure **20** is optionally employed to restrict solid support **18** to body **12**.

According to another aspect of the present invention there is provided a method of capturing and thereby removing particles characterized by surface exposure of anionic phospholipids present in biological fluids such as blood or blood-derived products. The method according to the present invention is effected by directing the biological fluid, such as blood or blood-derived products through an affinity filter, such as affinity filter **10** described hereinabove, to thereby remove, by affinity binding, the particles from the blood or blood-derived products, or other biological fluid.

As used herein in the specification and in the claims section below, the term "linked" refers to a direct linkage or an indirect linkage, effected by a spacer moiety. In both cases, anchoring can be effected either covalently or alternatively via an anchoring group characterized by non-covalent anchorage.

As used herein in the specification and in the claims section below, the term "affinity filter" also refers to "affinity column" or "affinity chromatograph".

As used herein in the specification and in the claims section below, the phrase "particles characterized by surface exposure of anionic phospholipids" includes apoptotic cells, apoptotic bodies, microemboli, cell debris, anionic phospholipid-exposing particles (APEP), membranes characterized in CMLA loss, and the like. It is clear that the terms recited in this paragraph are at least partially overlapping terms.

As further detailed hereinunder the anionic-phospholipid binding compound can be, yet it is not restricted to, a proteinaceous compound. As used herein, the term "proteinaceous compound" refers to a compound including, among optional additional components such as, but not limited to, lipids, a plurality of amino acid residues. The term "amino acid" is understood to include the 20 naturally occurring amino acid residues; those amino acid residues often modified post-translationally *in vivo*, including for example hydroxyproline, phosphoserine and phosphothreonine; and other unusual amino acid residues including, but not limited to, 2-aminoadipic acid, hydroxylysine, isodesmosine, nor-valine, nor-leucine and ornithine. Furthermore, the term "amino acid" includes both D- and L-amino acid residues. The amino acid residues according to the present invention form a peptide. The latter is understood to include native peptides, including degradation products or synthetically synthesized peptides, and further to peptidomimetics, such as peptoids and semipeptoids, which are peptide analogs, which may have, for example, modifications rendering the peptides more stable or less

immunogenic while contacting body fluids. Such modifications include, but are not limited to, cyclization, N terminus modification, C terminus modification, peptide bond modification, including, but not limited to, CH_2-NH, CH_2-S, CH_2-S=O, O=C-NH, CH_2-O, CH_2-CH_2, S=C-NH, CH=CH or CF=CH, backbone modification and residue modification. Methods for preparing peptidomimetic compounds are well known in the art and are specified in Quantitative Drug Design, C.A. Ramsden Gd., Chapter 17.2, F. Choplin Pergamon Press (1992), which is incorporated by reference as if fully set forth herein.

As used herein in the specification and in the claims section below, the term "solid support" is understood to include solid matrix, insoluble matrix and insoluble support.

An important aspect of the present invention is to provide such an affinity filter which functions as hereinabove described, while substantially maintaining the integrity of intact blood cells flowing therethrough. To this end, body **12** and solid support **18** are selected so as to allow the blood or blood-derived products to flow from inlet **14** through body **12** to outlet **16** at a flow rate and under pressure applicable for extracorporeal circulation, while substantially maintaining the integrity of intact blood cells flowing therethrough.

To this end, different types of solid supports can be used, examples include, but are not limited to, organic solid supports and inorganic solid supports. Regardless of the nature of the solid support, the anionic-phospholipid binding compound is immobilized or linked onto the solid support. Immobilizing or linking can be effected by physical interactions, ionic interactions or covalent link between the anionic-phospholipid binding compound and the solid support.

Thus, the solid support according to the present invention can be a stack of microparticulates, microfibers, microcapillaries or nanoparticles of, for example, alumina, diatomaceous earth, celite, calcium carbonate, calcium sulfate, ion-exchange resin, silica gel, charcoal, magnetic beads, amberlite, dowex, Eupergit and ethylsolfoxycellulose, all of which have been successfully used in the past to effectively link active proteins thereto.

According to preferred embodiments of the present invention, the solid support features a plurality of beads to which the anionic-phospholipid binding compounds are bound. Preferably, the beads are resin coated beads. Alternatively, the beads are magnetic beads which are most preferably microparticles or nanoparticles.

Alternatively, the filter can include a plurality of fibers or microcapillaries, among and/or through which the blood flows, the inner and/or outer faces thereof are covered with the anionic-phospholipid binding compound.

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According to a preferred embodiment of the present invention the anionic-phospholipid binding compound is an annexin or a portion thereof effective in binding the particles characterized by surface exposure of anionic phospholipids and thereby in removing the particles from the blood or blood-derived products. Specific binding to anionic phospholipid membranes is a common denominator of members of the annexin family of proteins, including, but not limited to, annexin I, annexin II, annexin III, annexin IV, annexin VI, annexin VII, annexin VIII, annexin XI and annexin XIII.

However, the annexin employed for binding the particles characterized by surface exposure of anionic phospholipids according to the present invention is preferably annexin V, which has some advantages in this respect over other annexins.

In any case, the annexin selected can be either recombinant or natural. By recombinant it is meant annexin purified from genetically modified cells, such as eukaryotic or prokaryotic cells, incorporating an active nucleic acid sequence encoding the annexin of choice. By natural it is meant annexin purified from a tissue of a living organism naturally producing annexin, e.g., placenta.

According to another preferred embodiment of the present invention, the anionic-phospholipid binding compound is of a general formula:

$$X_1\,[(X_3)_a / (X_4)_b]$$

wherein:

a and b are each independently an integer between 1 and 8;

X_1 is selected from the group consisting of a saturated fatty acid residue of 6 - 20 carbon atoms, an unsaturated fatty acid residue of 6 - 20 carbon atoms, a polyunsaturated fatty acid residue of 6 - 20 carbon atoms, and a cysteine residue bound through a thioether bond to a prenyl group of 5 - 20 carbon atoms, said residue being linked to an adjacent component of the compound through an amide bond;

X_3 comprises 1-6 amino acid residues, of which at least one amino acid residue is positively charged, wherein the remaining amino acid residues being polar uncharged amino acid residues; and

X_4 comprises 1-6 amino acid residues, of which 1-2 are aromatic amino acid residues, the remaining amino acid residues are selected from the group consisting of polar uncharged amino acid residues and hydrophobic (aliphatic) amino acid residues;

whereas, groups X_3 and X_4 present are optionally arranged in one of all possible linear

arrangements with respect to one another.

According to a preferred embodiment of the invention X_1 is a residue of a saturated fatty acid of a formula $CH_3(CH_2)_nCO_2H$,in which n is an integer between 8 - 18, or X_1 is a cysteine residue bound through a thioester bond to a prenyl of 5 - 15 carbon atoms.

According to another preferred embodiment of the invention X_1 is selected from the group consisting of myristic acid, palmitic acid and farnesyl cysteine.

According to yet another preferred embodiment of the invention the positively charged amino acid residues of X_3 are selected from the group consisting of lysine residue, arginine residue, histidine residue and an amino acid which includes a positively charged group covalently bound to an α-carbon atom or to an α-amine on a peptide backbone by a spacer selected from the group consisting of alkane or alkene of 1 - 4 carbon atoms and combinations thereof.

According to still another preferred embodiment of the invention the positively charged amino acid residues in X_3 are selected from the group consisting of lysine and arginine residues and combinations thereof.

According to an additional preferred embodiment of the invention the polar uncharged amino acid residues of X_3 are selected from the group consisting of serine, threonine, asparagine and glutamine residues and combinations thereof.

According to yet additional preferred embodiment of the invention the aromatic amino acid residues of X_4 are selected from the group consisting of phenylalanine and tryptophan residues and combinations thereof.

According to still additional preferred embodiment of the invention the polar uncharged amino acid residues of X_4 are selected from the group consisting of serine, asparagine and glutamine residues and combinations thereof.

According to another preferred embodiment of the invention the hydrophobic aliphatic amino acid residues of X_4 are selected from the group consisting of leucine, alanine and glycine residues and combinations thereof.

According to yet another preferred embodiment of the invention the anionic-phospholipid binding compound includes additional groups X_2, X_5 and X_6 and which has a general formula:

$$X_1\ X_2\ [(X_3)_a / (X_4)_b /(X_5)_c]\ X_6$$

wherein:

X_2 includes 0 - 3 glycine residues and 0 - 2 β-amino alanine molecules;

X_5 is selected from the group consisting of a linker βA-βA-P-βA-βA and a compound of a general formula:

wherein:

Z is a spacer group containing a carbon chain of 1 - 5 carbon atoms, saturated or unsaturated, substituted or unsubstituted, which is preferably selected from the group consisting of a saturated alkane and a non-saturated alkene;

J is a functional group selected from the group consisting of amines, thiols, alcohols, carboxylic acids, esters aldehydes and alkyl halides;

U is an anchoring group or an X_1 group;

c is an integer from 0 - 10; and

X_6 is zero or an X_1 group;

whereas, within subunit $[(X_3)_a/(X_4)_b/(X_5)_c]$ the groups X_3, X_4 and X_5 are arranged in one of all possible linear arrangements with respect to one another.

According to still another preferred embodiment of the invention X_5 is a lysine residue being substituted at an ε-amino group thereof by an anchoring group.

According to an additional preferred embodiment, the anionic-phospholipid binding compound is:

myristate-GGGKKKKKRFSFKKSFKLSGFSFKKNKKKU (SEQ ID NO:1),

myristate-GGGKKKKKRFSFKKSFKLSGFSFKKNKKK(biotin) (SEQ ID NO:1),

myristate-KKKKKRFSFKKSFKLSGFSFKKNKKKU (SEQ ID NO:2) or

myristate-KKKKKRFSFKKSFKLSGFSFKKNKKK(biotin) (SEQ ID NO:2).

According to alternative but preferred embodiments of affinity filter **10**, the anionic-phospholipid binding compound is a cyclized compound, which is preferably one of a group of compounds designated as "NST500 compound", which are described in greater detail with regard to Example 6 below.

As indicated in Figure 6, affinity filter **10** according to the present invention typically forms a portion (e.g., permanent, replaceable, add-on portion) of an extracorporeal circulation apparatus (also known in the art as, pump oxygenator apparatus, heart-lung machine, mechanical circulatory assistance and cardiopulmonary bypass), a blood transfusion apparatus,

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a hemodialysis apparatus or a plasmapheresis apparatus, all as indicated by pipe or tubing connections **24**. Thus, in the first case, for example, blood entering filter **10** originates from the patient itself, whereas, in the second case, blood or blood-derived products entering filter **10** originate from a container including same. The construction and operation of extracorporeal circulation and blood transfusion apparatuses are well known in the art and require no further description herein. Further details concerning extracorporeal circulation are found in Mora CT et al., (Editors): Cardiopulmonary bypass: Principles and Techniques of Extracorporeal Circulation. Springer Verlag, New York, 1995.

It will be appreciated by one ordinarily skilled in the art that for use in human beings, the affinity filter according to the present invention is preferably designed to be disposable. Measures are therefore preferably taken to provide for easy installation and removal of the filter when used with an apparatus having reusable components, such as an extracorporeal circulation apparatus.

The filter and method according to the present invention can be effectively applied to remove microparticles, such as microemboli and/or apoptotic debris in various situations and conditions, such as, but not limited to, during surgical operations, e.g., heart or lung surgeries, which call for extracorporeal circulation, in patients undergoing hemodialysis or plasmapheresis, in patients suffering from genetic blood or autoimmune diseases, such as, but not limited to diabetes, thalassemia and systemic lupus erythematosus, which are characterized by accelerated apoptosis of certain cell populations, and/or impairment of clearance of apoptotic bodies.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non-limiting fashion. These examples demonstrate the ability of NST300 compounds to bind to apoptotic cells. Since one of the hallmarks of apoptotic processes is early and prominent CMLA loss, these cells, therefore, serve as a well accepted and characterized model for CMLA loss, to thereby exemplify the ability of NST300

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compounds to bind particles characterized by surface exposure of anionic phospholipids. In addition, these examples demonstrate the utility and efficacy of NST300 compounds when bound to resin beads, as well as the efficacy and utility of NST500 compounds for the capture and remove of particles characterized by surface exposure of anionic phospholipids, and hence for the filtration of biological fluids containing such particles, particularly blood and blood-derived products.

EXAMPLE 1

Synthesis of NST300 compounds

Loading of Lys(Mtt) on solid support: 2.37 grams of 9- fluorenylmethoxycarbonyl (Fmoc)-Lys-(Mtt)-OH were dissolved in dichloromethane (DCM). 785 mg of N,N'dicyclohexylcarbodiimide (DCC) were added following the addition of 46 mg of dimethylaminopyridine (DMAP). Then, 2 grams of Wang resin (0.95 mmole/gram) were added and the reaction solution was stirred at room temperature for 2 hours. Then, the loaded resin was washed with DCM and N-methyl pyrrolidone (NMP) and then recoupled under the same conditions using half quantities of reagents. The resin was then washed and dried in vacuum. 3.18 grams of loaded resin were obtained.

Preparation of Lys(Biotin)-Resin: Fmoc-Lys(mtt)-Resin was stirred with a mixture of 1 % tri-fluoroacetic acid (TFA) and 0.1 % triisopropylsilane (TIS) in DCM at zero ^{o}C for 30 minutes and then for one hour at room temperature. Then, the resin was washed with NMP and DCM, and dried in vacuum. 2.68 grams of loaded resin were obtained. This resin was then swelled with 25 ml NMP in the presence of 930 mg Biotin, 1.44 grams O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), 513 mg N-hydroxybenzotriazole (HOBt) and 1 ml of diisopropylethylamine (DIEA). The reaction was stirred for 4.5 hours. Then, the resin was washed with NMP and DCM, and dried in vacuum. 2.8 grams of Fmoc-Lys(Biotin)-resin were obtained.

Preparation of myristate-GGGKKKKKRFSFKKSFKLSGFSFKKNK KK(Biotin)-OH (NST301, SEQ ID NO:1): Fmoc-Lys(Biotin)-Resin was used as starting material for the preparation of:

myristate-GGGKKKKKRFSFKKSFKLSGFSFKKNKKK(Biotin)-OH.

The synthesis was accomplished using an AB1 433A peptide synthesizer (Applied Biosystems UK) with HBTU/HOBt coupling reagents. Protected amino acid residues were introduced into the growing peptide-resin one after the other. The amino acid residues used

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were Fmoc-N^{α} protected. Trifunctional amino acid residues were side chain-protected as follows: Arg-2,2,5,7,8-pentamethyldihydrobenzofuran-5-sulfonyl(Pbf), Ser-tert-butyl(tBu), Lys-tertbutoxycarbonyl(Boc), Asn-trityl(Trt). Each Fmoc amino acid was activated in situ using a 1:1 HBTU/HOBt mixture and subsequently coupled to the resin for 50 minutes. DIEA was used during coupling as a non-nucleophilic base. The Fmoc protecting group on the amine was then removed with 20 % piperidine in NMP for 20 minutes. Three equivalents of the activated amino acid residues and coupling reagents (HBTU and HOBt) were employed in the coupling reactions. The deprotection and coupling steps were repeated with the addition of each subsequent amino acid until the peptide synthesis was completed. The final amino acid was deprotected using 20 % piperidine in NMP, and coupled with myristic acid under the same conditions as used for the introduced amino acid residues. The peptide-resin was washed with NMP, followed by DCM, and dried in vacuum. 562.5 mg of peptide resin were obtained.

Cleavage from the solid support: A cleavage mixture consisting of TFA 95 % and TIS 5% was added to the peptide-resin (20 ml of cleavage mixture per 1 gram resin). The solution was stirred at room temperature for 60 minutes. The resultant slurry (resin) was filtered using a sintered glass filter. The resin was washed twice with TFA. The filtrate was concentrated to a volume of 1 ml using a stream of nitrogen. Following the addition of cold diethyl ether (20 ml), the solution was cooled over an ice bath. After 60 minutes, the peptide was precipitated by centrifugation, washed with cold ether and dried under vacuum. 383.7 mg of crude peptide were obtained.

Purification and characterization: Peptide was purified by RP-HPLC on C_{18} 5μ of a Phenomenex Kromasil column (10 mm I.D. x 25 cm). Samples were eluted using the following gradient (i) distilled H_2O/0.05 % TFA, λ = 214 nm; and (ii) acetonitrile 0.05 % TFA, λ = 214 nm, flow 5 ml/minutes. The extent of purity of each peptide was monitored by rechromatography on C_{18} 5μ of Phenomenex Kromasil (4.6 mm I.D. x 25 cm) analytical column, flow 1 ml/minute. The characterization of the peptides was performed by Electrospray-Mass spectra (ES-MS). After purification, peptide was obtained at 91.5 % purity (non calibrated RP-HPLC, acetonitrile/water 0.1 % TFA gradient from 5 % to 50 % acetonitrile for 30 minutes. MS (ES) calculated: m/z for $C_{183}H_{304}N_{48}O_{38}S_1$ (MH+) 3814.3, found: 3816.2 (double charged).

Synthesis of NST302 and NST301-C: The same method as described for NST301 (SEQ ID NO:1) was also successfully and repeatedly used for the synthesis of NST302 (SEQ ID NO:2) and NST301-C (SEQ ID NO:3), respective of the appropriate sequence of each

compound. This further exemplifies the applicability of the above method of synthesis as a general method for synthesis of NST300 compounds. After purification, the NST302 peptide was obtained at 85.7% purity (non calibrated RP-HPLC, acetonitrile/water 0.1 % TFA gradient from 10 % to 35 % acetonitrile at 30 minutes. MS (ES) calculated: m/z for $C_{177}H_{295}N_{45}O_{35}S_1$ (MH+) is 3644.6, found: 3644.6 (double charged).

EXAMPLE 2

NST301 and NST302 bind to apoptotic cells

Re-distribution of anionic phospholipid molecules from the inner leaflet of the plasma membrane to the outer leaflet, is one of the early events occurring in apoptotic cells. The NST300 compounds are designed to bind membranes and fragments thereof characterized in CMLA loss. In order to test the ability of NST300 compounds to bind such membranes, the binding of NST301 and NST302 to cells undergoing apoptosis was measured.

Two modes of binding detection were used: the first mode demonstrates the binding to single cells by fluorescent microscopy, and the second mode demonstrates the binding to populations of cells by flow cytometric analysis.

Detection of binding of NST300 compounds to single apoptotic cells:

Fluorescent microscopy:

Compounds NST301 or NST302, labeled with biotin as a marker (at the X_5 domain, see Figures 1a-c) were used to study their binding to apoptotic cells. The compounds were each dissolved in TBS (Tris Buffered Saline; 10 mM Tris pH 8.0; 150 mM NaCl), at a stock concentration of 10 mM.

(a) Preparation of apoptotic cells: HeLa S3 cells (ATCC CCL-2.2) were cultured on a glass chamber slide (Nunc) on a culture area of 0.8 cm^3. Chamber slides were pre-coated with 1 % gelatin (Sigma). Cells were seeded at a density of 8 x 10^4 cells/chamber, in a volume of 300 µl of culture medium [Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM of L-Glutamine; 100 units/ml of Penicillin; 100 µg/ml of Streptomycin; 12.5 units/ml of Nystatin and 10 % of Fetal Calf Serum (FCS)]. Following 24 hours of incubation the cells were treated with the apoptotic trigger dopamine (DA), which is a well characterized model of apoptosis (Lou et al., J. Biol. Chem. 1998; 273:3756-3764). For the dopamine treatment, the culture medium was replaced by a low serum containing medium (2 % FCS), with 500 µM of dopamine (from RBI, MA, USA) for 18 hours.

(b) Evaluation of apoptosis: The evaluation was performed by staining with

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Hoechst 33342 dye (Molecular probes). This blue fluorescent dye is rapidly permeable into cells and stains DNA. Hoechst 33342 was added to growing cultures at a concentration of 1 μg/ml and 20 minutes later, the cells were visualized under UV light microscopy. The relative number of apoptotic cells with condensed or fragmented chromatin was then evaluated and compared with non-apoptotic cells which characteristically show a pale and diffuse staining. Photomicrographs were taken for documentation. An example of detection of apoptosis by Hoechst 33342 staining can be seen in Figure 2c. This method was used to evaluate the level of apoptosis in cultured cells prior to binding to the NST300 compounds. Cultures that exhibited at least 50 % of apoptosis following DA treatment were taken for further analysis.

(c) ***Binding of NST301 and NST302 to cells undergoing apoptosis:*** Apoptotic, as well as control untreated cells were grown as specified above and washed twice with TBS. The slides were then incubated with the NST300 compound at concentrations of 250 - 750 nM for 60 minutes, in a total volume of 100 μl. Slides were then dipped into a Couplin Jar containing 50 ml of TBS, and then incubated with 50 ng/ml of a streptavidin reagent labeled with FITC (Fluorescein Isothiocyanate Conjugated, from Jackson Immunolaboratory U.S.A.). Incubations were performed for 15 minutes at room temperature, in a final volume of 100 μl. The slides were then washed with TBS as above and were then mounted with Fluoroguard antifade reagent (from Biorad CA, USA). Binding was then evaluated with a fluorescent microscope (IX70; Olympus), using a NIBA filter (Narrow Interference Blue A from Olympus).

Figures 2a-c demonstrate the DA-induced apoptotic process in HeLa cells, and exhibit a typical culture with 50 % of the cells undergoing apoptosis, as can be identified by Hoechst 33342 staining. Figures 2d-c demonstrate the binding of the NST301 compound to cells undergoing dopamine - induced apoptosis. Strong staining of cells is evident. Figures 2f-g show staining of apoptotic cells in various stages of the death process. Staining ranged from mild peripheral membrane staining of the cells in the early stages of apoptosis to intense staining of the more-advanced apoptotic cells. The NST302 compound exhibited a similar staining profile. Non-treated cells served as control cells and did not show significant labeling by NST compounds. MST301-C served as a control compound. This compound did not show any significant binding to the cells.

This example shows that the NST300 compounds can serve to bind apoptotic cells.

Detection of binding of NST300 compounds to apoptotic cells by FACS analysis

(a) ***Preparation of apoptotic cells for FACS analysis:*** HeLa cells were plated on tissue culture plates at a density of 3×10^6 cells/10 cm dish, and grown in a DMEM medium

containing 10 % FCS, as described above. The cells were then incubated at 37 °C overnight, and then the medium was replaced with a medium containing 2 % FCS and 500 μM of dopamine. 18 hours later, the medium was aspirated and discarded, and 10 ml of Phosphate Buffered Saline (PBS), pH 7.4, were added to the culture dish. The cells which were detached from the culture dish into the PBS were collected. These cells represent advanced apoptotic cells. The remaining cells which were attached to the culture dish were trypsinized by the addition of 2 ml of trypsin for 2 minutes at 37 °C, followed by the addition of 10 ml of a medium containing 10 % of FCS. The cells were then washed with PBS containing 2 % Bovine Serum Albumin (BSA), and resuspended in TBS containing 2 % BSA (TBS-BSA). These cells were regarded as early apoptotic cells. Non-treated cells served as controls, and were subjected to similar treatments as above. Only non-treated cells that were attached to the culture dish were used as control cells for the FACS analysis.

(b) Binding of NST compounds to cells undergoing apoptosis: preparation for FACS: The binding of compounds (NST301 and NST302) and of the control compound (NST301-C) to samples of 5×10^5 cells was tested. A set of 3 different cell types was taken (i) control non-treated cells; (ii) early apoptotic cells; and (iii) advanced apoptotic cells. Each of the three types of cells was tested for binding to (i) compounds NST301 or NST302; (ii) control compound NST301-C; and (iii) no compound (FITC labeled streptavidin reagent only).

The incubation with the NST300 compounds or control compound was performed in a final volume of 100 μl TBS-BSA, 5 μg/ml of propidium iodide (PI) and 750 nM of the NST compound or control compound.

PI is a red fluorescent dye that stains DNA. It does not cross the plasma membrane of cells that are viable or cells that are in the early stages of apoptosis, since they maintain the plasma membrane integrity. Only cells that are in advanced apoptotic stages, or cells that have already died, are permeable to PI and are stained with the dye. The reactions were incubated at room temperature for 15 minutes and then collected by centrifugation at 1000 x g for 3 minutes. The cells were then washed in 500 μl of cold TBS-BSA and centrifuged as before. The cells were then suspended in 100 μl of TBS-BSA containing streptavidin conjugated to fluorescein (FITC). The incubation was performed for 15 minutes at room temperature in the dark. Thereafter, the cells were washed three times in TBS-BSA and were taken for FACS analysis.

(c) FACS analysis: The FACS analysis was performed on a Beckton-Dickinson cell sorter, using lysis II software. Excitation was at 488 nm and the emission for FITC detection was at 535 nm and for PI detection at 585 nm.

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Figure 3a demonstrates the binding of NST300 compounds to two different cell populations: early apoptotic cells, in comparison to control non-apoptotic cells. When each one of these cell populations was stained by FITC and PI only with no compound (indicative of cells that have already died via apoptosis), an increase of PI staining is observed as a function of advancement of the apoptotic process.

Staining with FITC-labeled NST301 demonstrated that the population of early apoptotic cells was stained strongly with the compound. Only 43 % of these cells were double stained also with PI, (indicative of advanced apoptotic cells).

Staining with the control compound NST301-C indicated residual binding of this compound to either one of the two different cell populations, similarly to the control examples of staining with PI only and with FITC only, but without the compound. These results indicate that NST301 can specifically bind to apoptotic cells at the early stages of the death process, and therefore can be used as detector of early apoptosis.

The NST302 compound was used in similar binding experiments in order to determine its binding to apoptotic cells, and in order to compare its performance to the binding of NST301. When apoptotic cells were exposed to 750 nM of each one of the NST compounds, a higher binding intensity (measured as the FITC mean value) was measured for NST302 (Figure 3b) indicating that NST302 is more potent than NST301 in binding to apoptotic cells.

The ability of the NST301 compound to bind to populations of early apoptotic cells was further emphasized by the analysis performed in Figure 3c, in which the ratio between total FITC binding versus total PI binding was used as a variable to define the potential of the NST301 compound to bind to early apoptotic cells. A high FITC/PI ratio thus indicates that most of the cells in a given population bind NST301 whilst their plasma membrane is still intact. Early apoptotic cells had a higher FITC/PI value (2.2) as compared to a population of advanced apoptotic cells (having a value of 1.2). Theses values were dramatically higher than the FITC/PI values obtained for the same cellular populations when exposed only to PI and FITC, or when exposed to PI and the control compound NST300-C (Figure 3c). These data therefore further exemplify the potency of the NST301 compound to bind to cells at early apoptotic stages.

EXAMPLE 3

NST301 and NST302 compounds as potent anticoagulants

Inhibition of clotting induced by negatively charged phospholipids: In physiological conditions, as well as in a standard coagulation assay, anionic phospholipid molecules, and specifically PS, serve as a potent catalytic surface on which binding of various coagulation factors takes place, thus among others catalyzing the assembly of the prothrombinase complex (Mann KG, et. al. Blood 1990; 76:1-16).

The ability of NST300 compounds to inhibit coagulation catalyzed by negatively charged phospholipids was evaluated in a standard coagulation test of the Russell viper venom (RVV) assay. The RVV reagent directly activates factor X present in the plasma, thus promoting prothrombinase complex formation. This reagent is widely used as a standard phospholipid responsive clotting test. (Thiagarajan et al., 1986).

The RVV reagent kit containing RVV and negatively charged phospholipids, from Gradipore, Australia was used. The reactions were started by mixing 100 µl of RVV reagent, and 100 µl of quality control plasma collected from normal individuals (commercially available from Instrumentation Laboratory, Italy). Clotting time was determined as the time-point beyond which the continuously mixed reaction ingredients in the test tube could no longer be aspirated with a Pasteur pipette. Clotting time was measured independently by two separate individuals.

Clotting time was measured following addition of NST301 or NST302 compounds (at concentrations between 0.5 - 50 µM) to the above reaction mixture.

Figure 4 demonstrates the effect of NST300 compounds on the clotting time as measured in the above RVV test. A concentration-dependent binding curve for each one of the compounds is shown. The control clotting time, when no NST compound was added to normal plasma was 40 seconds. NST301 and NST302 markedly and significantly increased the clotting time by a factor of 2.6 and 3.1, respectively, as compared to control. ($p < 0.001$, Student's test).

EC_{50} (effective dose of 50 %) for these compounds in the paradigm used in this experiment was 5-10 micromolar.

A moderate effect was also observed with the NST301-C compound. NST302 was more potent as an anticoagulant than NST301. These experiments therefore show that NST300 compounds are potent anticoagulants, as they bind to membranes having exposed negatively charged phospholipids.

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EXAMPLE 4

NST300 compounds potently correct the procoagulant effects of apoptotic cells

During the early stages of apoptosis, loss of plasma membrane asymmetry occurs, leading to the exposure of anionic phospholipids in the outer plasma membrane. As a result, apoptotic cell surfaces can serve as procoagulants (Casciola-Rosen et al., Proc. Nat. Acad. Sci. 1996;93:1624-1629; Flynn PD et al., Blood 1997; 89:4378-4384). The procoagulant activity of apoptotic cells was demonstrated using a modified APTT (Activated Partial Thromboplastin Time) coagulation assay.

In the standard APTT test, clot formation is triggered by recalcification of plasma and addition of cephalin, i.e., negatively charged phospholipids. Time until clot formation was measured as described in Example 3 above. When normal control plasma was used, clotting was observed after 40 seconds. In the modified APTT test, used while reducing the present invention to practice, the addition of negatively-charged phospholipids (cephalin) was replaced by addition of cells.

HeLa S3 cells were treated with 500 μM of dopamine for 18 hours. The cultured medium was discarded, and cells that were loosely attached to the growing surface, were collected in PBS, as described in Example 2 above. These cells were washed and resuspended in TBS, and were regarded as advanced apoptotic cells. Equal numbers of apoptotic or control non-treated cells (10^5) in a volume of 100 μl, were mixed with 100 μl of 25 mM of $CaCl_2$ and the clotting time was measured. Mean clotting time in the presence of the control, non-apoptotic cells was 78 ± 1.4 seconds (mean ± SD). The apoptotic cells were highly procoagulant, shortening clotting time to 38.0±2.8 sec. These results demonstrate that apoptotic cells are highly procoagulant.

Inhibition of this procoagulant activity by NST300 compounds was tested following pre-incubation of the apoptotic cells with the different NST300 compounds.

Pre-incubation of equal numbers of apoptotic or control non-treated cells (10^5) with NST300 compounds was for 10 minutes at room temperature in a final volume of 100 μl. NST300 compounds were used at concentration of 0.5 μM. The results are shown in Figure 5.

The addition of NST301 or of NST302 compounds at a concentration of 0.5 μM to apoptotic cells increased the clotting time by a factor of 2, and corrected the procoagulant effect of the apoptotic cells, thus demonstrating the potential of NST300 compounds as potent inhibitors of this effect of apoptotic cells.

Conversely, the control peptide (NST301-C) had only a mild effect on the clotting time

in the presence of apoptotic cells.

EXAMPLE 5

NST300 compounds attached to beads provide active filtration

As previously described, anionic phospholipid molecules become redistributed from the inner leaflet of the plasma membrane to the outer leaflet in apoptotic cells. The compounds of the present invention, such as the NST300 compounds and the NST500 compounds (see Example 6 below), are designed to bind membranes and fragments thereof characterized by CMLA loss, thereby capturing apoptotic cells and cell-derived particles. Preferably, the ability of the compounds of the present invention, to capture these cells and cell-derived particles which are characterized by surface exposure of anionic phospholipids, is used to capture and remove material from biological fluids, such as blood and blood products.

The previous examples showed that the NST300 compounds of the present invention feature sufficiently strong binding capabilities to be useful in such a filter for biological fluids. A particularly preferred embodiment of the affinity filter of the present invention is now described with regard to experimental results in this Example, in which the solid support for attaching the compounds of the present invention features a plurality of beads.

Preferably, the beads are resin coated beads. Alternatively, the beads are magnetic beads which are most preferably microparticles or nanoparticles. Although the affinity filter of the present invention is described with regard to certain specific anionic-phospholipid binding compounds being attached to the beads, it is understood that substantially any such compound could be used with the beads in the affinity filter of the present invention, including but not limited to, the NST300 group of compounds; the NST500 group of compounds (see Example 6); and annexin or a portion thereof, for example, annexin V.

In order to demonstrate the utility of this preferred embodiment, the NST302 compound of the present invention was synthesized directly onto resin beads, it being understood that such beads are only an example of a bead material which could be used with the filter of the present invention and is not intended to be limiting in any way. Next, these prepared beads were tested for their ability to bind apoptotic cells, both with fluorescence microsopy (Figure 7) and confocal microscopy (Figure 8). In addition, the beads were shown to separate apoptotic, procoagulant cells from a mixture of cells and hence to increase the coagulation time of apoptotic cells (Figure 9). The experimental method was as follows.

Synthesis of NST302 onto Resin Beads

NST302-coated resin beads (B-302) were prepared as follows. Peptide synthesis was performed directly onto the resin beads with an ABI 433A peptide synthesizer with HBTU/HOBt coupling reagents. The peptide was synthesized such that it was linked to the resin beads through the linker βA-βA-P-βA-βA, such that the linker was synthesized as part of the peptide at the C-terminus. Amino acids used were Fmoc-N^{α} protected. Trifunctional amino acids were side chain protected as follows: Ser(tBu), Lys(Boc), Asn(Trt), and Glu(OtBu). 400 micromoles of TentaGel Resin (resin beads) were placed in the reaction vessel. Each Fmoc amino acid was activated *in situ* by using TBTU/HOBt and was subsequently coupled to the resin for 50 minutes. DIEA was used during coupling as a non-nucleophilic base. The Fmoc protecting group on the α amine was then removed with 20% piperidine in NMP for 20 minutes. 2.5 equivalents of the activated amino acids were employed in the coupling reactions. The deprotection and coupling steps were repeated with the addition of each subsequent amino acid until the peptide synthesis was completed. The final amino acid was deprotected using 20% piperidine in NMP, and coupled with myristic acid under the same conditions as with the amino acids. The peptide-resin was washed with NMP, followed by DCM and dried under vacuum.

A cleavage mixture consisting of TFA 95% and TIS 95% was added to the peptide-resin (20 microliters of cleavage mixture to 1 gram of resin), in order to cleave the protecting groups from the synthesized peptide. The solution was set to vortex at room temperature for 60 minutes. The resultant slurry (resin) was filtered with a sintered glass filter. The resin was washed twice with TFA and DCM and dried under vacuum. Then, 10 ml of water was added and the peptide-resin was lyophilized.

Binding of Apoptotic Cells to NST302-coated Beads

The capability of resin coated beads (B-302), prepared as described above, to bind apoptotic cells selectively was examined. Apoptotic HeLa cells (treated for 16 hours with 500 micromolar dopamine) were incubated with TentaGel beads (control beads) or with beads coated with NST 302 through a linker (B-302) at a ratio of approximately 1/50 beads/cells. The mixture was stained with Hoechst 33342 and then the beads were separated from the unbound cells and were photographed in a fluorescent microscope (Figure 7). The B-302 beads which were incubated with apoptotic cells bound many cells to the surface of the bead. However control beads remained free of cells, indicating that the apoptotic cells specifically bound to the

B-302 beads through the NST302 molecules, rather than from binding non-specifically to the bead material itself (Figure 7).

With confocal microscopy, an entire bead with any bound cells can be visualized after staining with Hoechst 33342 (Figure 8). As shown in the confocal microscopy photograph, the number of cells which are bound to each B-302 bead was found to be about 10-20 cells, while the control beads remained essentially free of cells. Thus, both confocal and fluorescence microscopy showed that the apoptotic cells bound specifically to the B-302 beads, due to the presence of the NST302 compound of the present invention on the bead surface, and did not bind to the control beads, which lacked the NST302 compound.

Furthermore, these results demonstrate that an affinity filter according to the present invention could usefully feature such beads coupled to a compound of the present invention such as an NST300 compound such as NST302, or to an annexin substance such as annexin V.

NST302-coated Beads Reduce the Procoagulant Activity of Apoptotic Cells

One of the earlier steps in the process of apoptosis is the loss of membrane asymmetry and exposure of PS (phosphatidylserine). As previously described, the loss of membrane symmetry and the exposure of PS can serve as a surface for the binding of clotting factors and therefore are procoagulant. Hence, treatment of apoptotic cells with B-302 may capture these cells and thereby decrease the ratio of apoptotic cells, leading to an increase in coagulation time.

In order to demonstrate that such treatment is useful to increase coagulation time, apoptotic HeLa cells (treated with 350 micromolar dopamine) were incubated with B-302 or control beads, and the coagulation time catalyzed by the apoptotic cells was measured in a modified APTT test. HeLa cells (ATCC CCL-2.2) were normally grown in DMEM supplement with 10% fetal calf serum. Apoptosis was induced by growing the cells for 16-20 hrs. in medium containing 2% fetal calf serum in the presence of 350 or 500 micromolar dopamine. Cells from 100 mm tissue culture plate were scraped in buffer containing 150 mM NaCl and 10 mM Hepes, pH 7.1 (HBS). Cells were washed once and resuspended in 1 ml and the number of cells was determined. TentaGel resin beads and beads coated with NST302, attached through the linker βA-βA-P-βA-βA (B-302), were produced as previously described.

Beads were washed several times with HBS and resuspended at 40 mg/ml (4-6x10^4 beads/ml). Separation of the cells from the beads was done by simple gravitational precipitation of the beads for a few seconds and pipetting out the cells followed by washing the beads with

200 microliters of buffer, which was added to the cell suspension. For nuclear staining, Hoechst 33342 (molecular Probes) was added to a final concentration of 1 microgram/ml. Coagulation test was done with IL Test (Instrumentation Laboratory, MA, USA) as follows: 50 microliters of a cell suspension were added to 100 microliters of normal plasma. Coagulation was initiated by adding 80 microliters of 25 mM $CaCl_2$ and the time until clot formation was measured. The mean time of triplicate tests +/- SD is shown.

As shown in Figure 9, treatment with B-302 beads clearly increased the coagulation time with apoptotic cells by 84%, as compared to cells treated with control beads. These results suggest that B-302 beads bind apoptotic cells selectively, and separate them from non-apoptotic and non-procoagulant cells. Thus, beads coupled to a compound according to the present invention, such as an NST300 compound such as NST302 for example, were clearly shown to be useful to inhibit the coagulation process mediated by apoptotic cells and thus to increase coagulation time with apoptotic cells.

EXAMPLE 6

NST500 compounds provide excellent binding properties

NST500 compounds are a second novel group of compounds, described in Israeli Patent Application No. 131266, filed on August 5, 1999, which are capable of high-affinity binding to anionic phospholipid membranes. Although such NST500 compounds have certain functional capabilities with regard to filtration of biological fluids which are similar to those capabilities of the NST300 compounds, NST500 compounds are structurally very different, and feature improved binding characteristics. Thus, the NST500 compounds represent an improved group of compounds, particularly in terms of their higher overall activity, stability and ability to provide high-affinity binding to anionic phospholipid membranes, for example for the filtration of biological fluids, particularly blood and blood-derived products.

As previously mentioned, the NST500 compounds may be generally described as also including a cyclized form of the compounds of general formula I or Ia, such that the cyclized compound features at least a fatty acid moiety and a plurality of amino acid residues. However, the preferred NST500 compounds are compounds of a general formula III:

X_1-X_2-{$(X_3)_a$/X_4/X_5}-X_6

wherein:

X_1 stands for a saturated or unsaturated fatty acid residue comprising 6 - 20 carbon atoms; or a cysteine residue bound through a thioether bond to a prenyl group

comprising 5 - 20 carbon atoms; the residue being linked to the adjacent component of the compound through an amide bond;

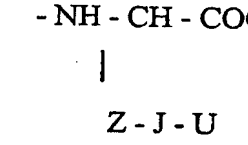
X_2 is either 0 or includes at least one unit of a general formula (IV) $A*2^n$, in which A stands for a branching unit and n stands for 0 - 4;

X_3 comprises 1-6 amino acids, of which 1-6 are positively charged and 0-2 are negatively charged, any remaining amino acid residues being polar uncharged amino acids;

X_4 comprises 1-6 amino acids, of which 1-2 are aromatic amino acids, any remaining amino acids being selected among polar uncharged amino acids and hydrophobic aliphatic amino acids;

X_5 comprises 1-6 amino acids, of which 1-6 are positively charged and 0-2 are negatively charged, any remaining amino acid residues being polar uncharged amino acids, wherein the amino acids have a cyclic structure;

X_6 is selected from the group consisting of a linker, such as βA-βA-P-βA-βA for example, or a compound of general formula II



wherein Z stands for a spacer group containing a chain of 1 - 5 carbon atoms, saturated or unsaturated, substituted or unsubstituted, preferably alkane or alkene; J represents a functional group selected among amines, thiols, alcohols, carboxylic acids and esters, aldehydes and alkyl halides; U is either 0 or a labeling moiety;

wherein:

a is an integer of 1 - 3; and

the groups X_3, X_4 and X_5 are each located at an individually selected location in the compound, such that one of any possible linear combination of the groups X_3, X_4 and X_5 is selected;

as well as functional equivalents thereof and/or compounds having the same biological activity thereto.

A particularly preferred but exemplary compound of a general formula III is:

Myristate - KVSFFCKNKEKKC – K-βA-βA-P-βA-βA, in which the two cysteine residues are linked via a disulfide bond, to form a cyclic structure, wherein K=lysine, V=Valine, S=serine, F=phenylalanine, C=Cysteine, N=asparagine, E=Glutamic acid and the compound features the linker βA-βA-P-βA-βA, through which the compound is linked to a resin bead. This compound is

herein called "NST513-B" and is featured in the experimental data which is described below. Although as shown, NST513-B has marked and substantial activity when linked to the resin bead through the linker βA-βA-P-βA-βA, this compound of the present invention is understood not to be limited to such an embodiment, and may also be prepared without the linker.

As described in greater detail below, the particularly preferred compound according to the present invention, NST513-B, was synthesized and tested for the ability to selectively bind to activated platelets. As one of the known features of platelet activation is the redistribution of phosphatidylserine (PS) from the inner to the outer leaflet of the platelet membrane, NST513-B was shown to be able to bind to such activated platelets, presumably by binding to PS, although the present invention is not restricted to any particular mechanism of action. NST513-B was synthesized onto resin beads, in a synthetic procedure similar to that described for NST302 in Example 5 above and as described in further detail below. The activated platelets were washed and then incubated with the NST513-B-coated beads. The experimental method was as follows.

Synthesis of NST 500 cyclic compounds

A general description of the preparation of the N-terminal myristoylated, biotinylated cyclic peptide according to the present invention is as follows. Orthogonally protected diaminoacid was loaded on a solid support. Then the α-amino protecting group was removed and the βA-βA-P-βA-βA linker was added, followed by the sequence of NST513. Then, the α-amino protecting group was removed and the peptide was prepared sequentially on the solid support.

Following the removal of the α-amino protecting group of the N-terminal amino acid, myristic acid was introduced to the peptide-resin under conditions which were similar to those used during the coupling of the amino acids. Then, the peptide was cleaved from the solid support, purified and characterized using HPLC/MS.

Method of Cyclization: Cyclization of the peptide via an intramolecular di-sulphide bridge was formed by air oxidation, iodine treatment, removal of S-Acm protecting groups on the Cysteine residues with mercury acetate followed by air oxidation, or simultaneous deprotection/oxidation with $TI(TFA)_3$. Cyclization was also carried out by an amide bond which was formed either while the peptide was still attached to the resin, or in solution, as follows:

An amine and a carboxyl side chains were protected by Fmoc and Ofm protecting groups or by Allyl and Alloc protecting groups respectively, during the synthesis. While the peptide-

resin was attached to the resin, these protecting groups were removed and an amide bond was formed using an appropriate coupling reagent. Then, the peptide was purified, lyophilized and characterized by HPLC/MS.

Synthesis of Myristate-KVSFFCKNKEKKCK-βA-βA-P-βA-βA

A preferred synthetic procedure for the preparation of NST513-B is given below, in which the peptide was synthesized directly onto the resin beads. The peptide was synthesized such that it was linked to the resin beads through the linker βA-βA-P-βA-βA, such that the linker was synthesized as part of the peptide at the C-terminus. Alternatively, the peptide could be synthesized without the linker to form NST513.

Loading of Lys(Mtt) on a solid support

2.5 gr Fmoc-Lys(Mtt)-OH was dissolved in 150 ml NMP.620 mg of HOBt was added following the addition of 1.3 gr of TBTU. Then, 3.5 gr of Rink amide resin (0.58 mmole/gr) was added and the reaction was stirred at room temperature for 3.5 hr. Then, the loaded resin was washed with NMP, MeOH, DCM and then dried in vacuum. 4.4 gr. of loaded resin was obtained.

Synthesis of the peptide backbone

Peptide synthesis was accomplished using an ABI 433A peptide synthesizer with HBTU/HOBt coupling reagents, such that the peptide was synthesized directly onto the resin bead with the linker βA-βA-P-βA-βA, such that the linker was synthesized as part of the peptide at the C-terminus and was also attached to the resin bead. Amino acids used were Fmoc-Nα protected. Trifunctional amino acids were side chain protected as follows: Ser(tBu), Lys(Boc), Asn(Trt), Glu(OtBu). 400 micromol of Preloaded Lys-Resin was placed in the reaction vessel. Each Fmoc amino acid was activated in situ using TBTU/HOBt and subsequently coupled to the resin for 50 min. DIEA was used during coupling as a non nucleophilic base. The Fmoc protecting group on the α amine was then removed with 20% piperidine in NMP for 20 min. 2.5 equivalents of the activated amino acids were employed in the coupling reactions. The deprotection and coupling steps were repeated with the addition of each subsequent amino acid until the peptide synthesis was completed. The final amino acid was deprotected using 20% piperidine in NMP, and coupled with myristic acid under the same conditions as with amino acids introduced. The peptide-resin was washed with NMP, followed by DCM, and dried in vacuum.

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Cleavage of protecting groups

A cleavage mixture consisting of TFA 95% and TIS 5% was added to the peptide-resin (20 ml cleave mixture to 1 gr. resin). The solution was stirred at room temperature for 60 min. The resultant slurry (resin) was filtered using a sintered glass filter. The resin was washed twice with TFA and DCM, and then dried under vacuum. Then 10 ml of water was added and the peptide-resin was lyophilized.

Cyclization of the peptide attached to resin beads

Cyclization of the peptide-coated beads was performed either through air oxidation or by DMSO treatment. For air oxidation, the reaction mixture was stirred for 2 days, while air was bubbled through the solution. Then the solution was freeze dried and purified under similar conditions as described above. For DMSO treatment, the beads were suspended in 30 % DMSO for 24 hours at room temperature. The beads were then washed with water and methanol, and dried under vacuum.

NST513-B-coated beads selectively bind activated platelets

The resin beads which were coated with the NST513-B compound were then tested for their further ability to bind to activated platelets. As described previously, one of the known features of platelet activation is the redistribution of PS from the inner to the outer leaflet of the plasma membrane. Preliminary studies showed that NST513-B binds selectively to activated platelets. Therefore, the capability of NST513-B bound TentaGel beads (NST513-B) to bind and remove activated platelets from an equal mixture of activated and control platelets was tested, in order to demonstrate the utility of the NST500 compounds of the present invention for the filtration of biological fluids. The unbound cells were subjected to a further FACS analysis using biotinylated Annexin V and Streptavidin-FITC. Figure 10 depicts the results of a typical experiment showing a significant reduction in FITC positive platelets treated with NST513-B compared to control beads, thereby demonstrating that the NST513-B beads specifically captured activated platelets. The experimental method was as follows.

Platelets were obtained from the blood bank of the local medical center. The platelets were washed and resuspended in platelets buffer (137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.5 mM $NaHPO_4$, 5 mM glucose and 20 mM Hepes, pH 7). Platelets were diluted 10 fold and activated by incubation with 2 micromolar A23187 and 5 mM $CaCl_2$ for 10-15 minutes after they were washed again. Labeling with Annexin was done using Annexin V-biotin apoptosis

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detection kit (BioVision) and Streptavidin-FITC (Jackson Laboratories). The platelets were then incubated with control beads, 7.5 mg of NST513-B beads, or 25 mg of NST513-B beads.

As shown in Figure 10, the NST513-B beads captured activated platelets, such that the number of FITC-positive cells decreased significantly after incubation with the NST513-B beads. The effect was shown to be dose-dependent, in that 25 mg of the NST513-B beads had a much greater effect than 7.5 mg of the beads. By contrast, a much higher percentage of FITC positive cells remained after incubation with the control beads.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

WHAT IS CLAIMED IS:

1. An affinity filter effective in capturing and thereby removing particles characterized by surface exposure of anionic phospholipids present in a biological fluid, the affinity filter comprising a body containing a solid support and an anionic-phospholipid binding compound linked to said solid support, said anionic-phospholipid binding compound being for specifically binding the particles characterized by surface exposure of anionic phospholipids and thereby removing the particles from said biological fluid.

2. The affinity filter of claim 1, wherein said biological fluid is blood or a blood-derived product.

3. The affinity filter of claim 2, wherein said body and said solid support are selected so as to allow the blood or blood-derived products to flow through said body while substantially maintaining the integrity of intact blood cells flowing therethrough.

4. The affinity filter of claim 1, wherein said solid support is selected from the group consisting of an organic solid support and inorganic solid support.

5. The affinity filter of claim 1, wherein said solid support is formed of a structure selected from the group consisting of microparticulates, microfibers, beads and microcapillaries.

6. The affinity filter of claim 5, wherein said beads are selected from the group consisting of resin beads and magnetic beads.

7. The affinity filter of claim 6, wherein said magnetic beads are selected from the group consisting of microparticles and nanoparticles.

8. The affinity filter of claim 1, wherein said anionic-phospholipid binding compound is an annexin or a portion thereof effective in binding the particles characterized by surface exposure of anionic phospholipids and thereby removing the particles from said biological fluid.

9. The affinity filter of claim 8, wherein said annexin is selected from the group consisting of annexin I, annexin II, annexin III, annexin IV, annexin VI, annexin VII, annexin VIII, annexin XI and annexin XIII.

10. The affinity filter of claim 9, wherein said annexin is annexin V.

11. The affinity filter of claim 8, wherein said annexin is recombinant.

12. The affinity filter of claim 8, wherein said annexin is natural.

13. The affinity filter of claim 1, wherein said anionic-phospholipid binding compound is of a general formula:

$$X_1\ [(X_3)_a / (X_4)_b]$$

wherein:

a and b are each independently an integer between 1 and 8;

X_1 is selected from the group consisting of a saturated fatty acid residue of 6 - 20 carbon atoms, an unsaturated fatty acid residue of 6 - 20 carbon atoms, a polyunsaturated fatty acid residue of 6 - 20 carbon atoms, and a cysteine residue bound through a thioether bond to a prenyl group of 5 - 20 carbon atoms, said residue being linked to an adjacent component of the compound through an amide bond;

X_3 comprises 1-6 amino acid residues, of which at least one amino acid residue is positively charged, wherein the remaining amino acid residues being polar uncharged amino acid residues; and

X_4 comprises 1-6 amino acid residues, of which 1-2 are aromatic amino acid residues, the remaining amino acid residues are selected from the group consisting of polar uncharged amino acid residues and hydrophobic amino acid residues; whereas, groups X_3 and X_4 present are arranged in one of all possible linear arrangements with respect to one another.

14. The affinity filter of claim 13, wherein X_1 is a residue of a saturated fatty acid of a formula $CH_3(CH_2)_nCO_2H$,in which *n* is an integer between 8 - 18, or X_1 is a cysteine residue bound through a thioester bond to a prenyl of 5 - 15 carbon atoms.

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15. The affinity filter of claim 14, wherein X_1 is selected from the group consisting of myristic acid, palmitic acid and farnesyl cysteine.

16. The affinity filter of claim 13, wherein the positively charged amino acid residues of X_3 are selected from the group consisting of lysine residue, arginine residue, histidine residue and an amino acid which includes a positively charged group covalently bound to an α-carbon atom or to an α-amine on a peptide backbone by a spacer selected from the group consisting of alkane and alkene of 1 - 4 carbon atoms and combinations thereof.

17. The affinity filter of claim 16, wherein the positively charged amino acid residues in X_3 are selected from the group consisting of lysine and arginine residues and combinations thereof.

18. The affinity filter of claim 13, wherein the polar uncharged amino acid residues of X_3 are selected from the group consisting of serine, threonine, asparagine and glutamine residues and combinations thereof.

19. The affinity filter of claim 13, wherein the aromatic amino acid residues of X_4 are selected from the group consisting of phenylalanine and tryptophan residues and combinations thereof.

20. The affinity filter of claim 13, wherein the polar uncharged amino acid residues of X_4 are selected from the group consisting of serine, asparagine and glutamine residues and combinations thereof.

21. The affinity filter of claim 13, wherein the hydrophobic aliphatic amino acid residues of X_4 are selected from the group consisting of leucine, alanine and glycine residues and combinations thereof.

22. The affinity filter of claim 13, wherein the anionic-phospholipid binding compound includes additional groups X_2, X_5 and X_6 and which has a general formula:

$$X_1\ X_2\ [(X_3)_a / (X_4)_b / (X_5)_c]\ X_6$$

wherein:

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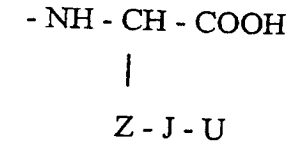
X_2 includes 0 - 3 glycine residues and 0 - 2 β-amino alanine molecules;

X_5 is selected from the group consisting of a linker βA-βA-P-βA-βA and a compound of a general formula:



wherein:

Z is a spacer group selected from the group consisting of a saturated alkane and a non-saturated alkene containing 1 - 5 carbon atoms;

J is a functional group selected from the group consisting of amines, thiols, alcohols, carboxylic acids, esters aldehydes and alkyl halides;

U is an anchoring group or said X_1 moiety;

c is an integer from 0 - 10; and

X_6 is zero or said X_1;

whereas, within subunit $[(X_3)_a/(X_4)_b/(X_5)_c]$ the groups X_3, X_4 and X_5 are arranged in one of all possible linear arrangements with respect to one another.

23. The affinity filter of claim 20, wherein X_5 is a lysine residue being substituted at an ε-amino group thereof by an anchoring group.

24. The affinity filter of claim 23, wherein said anionic-phospholipid binding compound is myristate-GGGKKKKKRFSFKKSFKLSGFSFKKNKKKU.

25. The affinity filter of claim 24, wherein said anionic-phospholipid binding compound is myristate-GGGKKKKKRFSFKKSFKLSGFSFKKNKKK(biotin).

26. The affinity filter of claim 23, wherein said anionic-phospholipid binding compound is myristate-GGGKKKKKRFSFKKSFKLSGFSFKKNKKK-βA-βA-P-βA-βA.

27. The affinity filter of claim 23, wherein said anionic-phospholipid binding compound is myristate-KKKKKRFSFKKSFKLSGFSFKKNKKKU.

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28. The affinity filter of claim 27, wherein said anionic-phospholipid binding compound is myristate-KKKKKRFSFKKSFKLSGFSFKKNKKK(biotin).

29. The affinity filter of claim 23, wherein said anionic-phospholipid binding compound is myristate-KKKKKRFSFKKSFKLSGFSFKKNKKK-βA-βA-P-βA-βA.

30. The affinity filter of claim 2, forming a portion of an extracorporeal circulation apparatus.

31. The affinity filter of claim 2, forming a portion of a blood transfusion apparatus.

32. The affinity filter of claim 2, forming a portion of a hemodialysis apparatus.

33. The affinity filter of claim 2, forming a portion of a plasmapheresis apparatus.

34. The affinity filter of claim 1, wherein said anionic-phospholipid binding compound is of a general formula (I):

$X_1 [(X_3)_a / (X_4)_b]$

wherein:

a and *b* are each independently an integer between 1 and 8;

X_1 is selected from the group consisting of a saturated fatty acid residue of 6 - 20 carbon atoms, an unsaturated fatty acid residue of 6 - 20 carbon atoms, a polyunsaturated fatty acid residue of 6 - 20 carbon atoms, and a cysteine residue bound through a thioether bond to a prenyl group of 5 - 20 carbon atoms, said residue being linked to an adjacent component of the compound through an amide bond;

X_3 comprises 1-6 amino acid residues, of which at least one amino acid residue is positively charged, wherein the remaining amino acid residues being polar uncharged amino acid residues; and

X_4 comprises 1-6 amino acid residues, of which 1-2 are aromatic amino acid residues, the remaining amino acid residues are selected from the group consisting of polar uncharged amino acid residues and hydrophobic amino acid residues; wherein said anionic-phospholipid binding compound is a cyclized compound.

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35. The affinity filter of claim 1, wherein said anionic-phospholipid binding compound is a compound of general formula III:

$$X_1\text{-}X_2\text{-}\{(X_3)_a/X_4/X_5\}\text{-}X_6$$

wherein:

X_1 stands for a saturated or unsaturated fatty acid residue comprising 6 - 20 carbon atoms; or a cysteine residue bound through a thioether bond to a prenyl group comprising 5 - 20 carbon atoms; the residue being linked to the adjacent component of the compound through an amide bond;

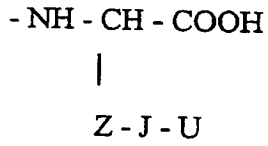
X_2 is either 0 or includes at least one unit of a general formula (IV) A^*2^n, in which A represents a branching unit and *n* is an integer of 0 - 4;

X_3 comprises 1-6 amino acids, of which 1-6 are positively charged and 0-2 are negatively charged, any remaining amino acid residues being polar uncharged amino acids;

X_4 comprises 1-6 amino acids, of which 1-2 are aromatic amino acids, any remaining amino acids being selected among polar uncharged amino acids and hydrophobic aliphatic amino acids;

X_5 comprises 1-6 amino acids, of which 1-6 are positively charged and 0-2 are negatively charged, any remaining amino acid residues being polar uncharged amino acids, wherein the amino acids form a cyclic structure;

X_6 is selected from the group consisting of a linker and a compound of general formula II



wherein Z stands for a spacer group containing a chain of 1 - 5 carbon atoms, saturated or unsaturated, substituted or unsubstituted; J represents a functional group selected among amines, thiols, alcohols, carboxylic acids and esters, aldehydes and alkyl halides; and wherein: *a* is an integer of 1 – 3.

36. The affinity filter of claim 35, wherein said chain for Z is an alkane or an alkene.

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37. The affinity filter of claim 35, wherein the groups X_3, X_4 and X_5 are each located at a separately selected location in the compound.

38. The affinity filter of claim 35, wherein X_1 is a residue of a saturated fatty acid of formula $CH_3(CH_2)_nCO_2H$, in which *n* is an integer of 8 - 18; or for a cysteine residue bound through a thioether bond to a prenyl group of 5 - 20 carbon atoms.

39. The affinity filter of claim 38, wherein X_1 is selected among myristic acid and palmitic acid; and farsenyl cysteine.

40. The affinity filter of any of claims 37 to 39, wherein branching unit A of X_2 is selected among a dicarboxylic and a poly-carboxylic acid.

41. The affinity filter of claims 37 to 40, wherein the positively charged amino acids of X_3 and X_5 are selected among lysine, arginine, histidine or any amino acid which is comprised of a positively charged group covalently bound to the α-carbon atom or to the α-amine on the peptide backbone by a spacer selected from a group comprised of an alkene of 1 - 4 carbon atoms and combinations thereof.

42. The affinity filter of claim 41, wherein the positively charged amino acids in X_3 and X_5 are selected among lysine and arginine and combinations thereof.

43. The affinity filter of any of claims 37 to 42 wherein the negatively charged amino acids of X_3 and X_5 are selected among glutamate and aspartate.

44. The affinity filter of any of claims 37 to 43 wherein the polar uncharged amino acids of X_3 and X_5 are selected among serine, threonine, asparagine and glutamine and combinations thereof.

45. The affinity filter of any of claims 36 to 44, wherein the aromatic acids of X_4 are selected among phenylalanine and tryptophan and combinations thereof.

46. The affinity filter of any of claims 37 to 45, wherein the polar uncharged amino

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acids of X_4 are selected among serine, asparagine and glutamine and combinations thereof.

47. The affinity filter of any of claims 37 to 46, wherein the hydrophobic aliphatic amino acids of X_4 are selected among leucine, valine, alanine and glycine and combinations thereof.

48. The affinity filter of any of claims 37 to 47, wherein the cyclization of X_5 is performed via an intra-molecular di-sulfide bridge or by an amide bond.

49. The affinity filter of claim 48, wherein X_6 stands for a cysteine residue bound through a thioether bond to a prenyl group, in which the cysteine carboxyl is either free or methylated.

50. The affinity filter of claim 35, wherein said compound of general formula III is Myristate - KVSFFCKNKEKKC - K.

51. The affinity filter of claim 35, wherein said compound of general formula III is Myristate - KVSFFCKNKEKKC – K and wherein the two cysteine residues are linked via a disulfide bond, to form a cyclic structure.

52. The affinity filter of claim 35, wherein said compound of general formula III is Myristate - KKVFSFCKNKEKKC- K.

53. The affinity filter of claim 35, wherein said compound of general formula III is Myristate - KKVFSFCKNKEKKC – K, wherein the two cysteine residues are linked together.

54. The affinity filter of any of claims 50 to 53, wherein said compound of general formula III further features said linker for linking said compound of general formula III to said solid support.

55. The affinity filter of claim 54, wherein said linker is βA-βA-P-βA-βA.

56. A method of capturing and thereby removing particles characterized by surface

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exposure of anionic phospholipids present in a biological fluid, the method comprising the step of directing the biological fluid through an affinity filter including a body containing a solid support and an anionic-phospholipid binding compound linked to said solid support, said anionic-phospholipid binding compound being for specifically binding the particles characterized by surface exposure of anionic phospholipids and thereby removing the particles from the biological fluid.

57. The method of claim 56, wherein said biological fluid is blood or a blood-derived product.

58. The method of claim 57, wherein said body and said solid support are selected so as to allow the blood or blood-derived products to flow through said body while substantially maintaining the integrity of intact blood cells flowing therethrough.

59. The method of claim 56, wherein said solid support is selected from the group consisting of an organic solid support and inorganic solid support.

60. The method of claim 56, wherein said solid support is formed of a structure selected from the group consisting of microparticulates, microfibers, beads and microcapillaries.

61. The method of claim 60, wherein said beads are selected from the group consisting of resin beads and magnetic beads.

62. The method of claim 61, wherein said magnetic beads are selected from the group consisting of microparticles and nanoparticles.

63. The method of claim 57, wherein said anionic-phospholipid binding compound is an annexin or a portion thereof effective in binding the particles characterized by surface exposure of anionic phospholipids and thereby removing the particles from the blood or blood-derived products.

64. The method of claim 63, wherein said annexin is selected from the group consisting of annexin I, annexin II, annexin III, annexin IV, annexin VI, annexin VII, annexin

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VIII, annexin XI and annexin XIII.

65. The method of claim 64, wherein said annexin is annexin V.

66. The method of claim 64, wherein said annexin is recombinant.

67. The method of claim 64, wherein said annexin is natural.

68. The method of claim 64, wherein said anionic-phospholipid binding compound is of a general formula:

X_1 $[(X_3)_a / (X_4)_b]$

wherein:

a and *b* are each independently an integer between 1 and 8;

X_1 is selected from the group consisting of a saturated fatty acid residue of 6 - 20 carbon atoms, an unsaturated fatty acid residue of 6 - 20 carbon atoms, a polyunsaturated fatty acid residue of 6 - 20 carbon atoms, and a cysteine residue bound through a thioether bond to a prenyl group of 5 - 20 carbon atoms, said residue being linked to an adjacent component of the compound through an amide bond;

X_3 comprises 1-6 amino acid residues, of which at least one amino acid residue is positively charged, wherein the remaining amino acid residues being polar uncharged amino acid residues; and

X_4 comprises 1-6 amino acid residues, of which 1-2 are aromatic amino acid residues, the remaining amino acid residues are selected from the group consisting of polar uncharged amino acid residues and hydrophobic amino acid residues; whereas, groups X_3 and X_4 present are arranged in one of any possible linear arrangement with respect to one another.

69. The method of claim 68, wherein X_1 is a residue of a saturated fatty acid of a formula $CH_3(CH_2)_nCO_2H$,in which *n* is an integer between 8 - 18, or X_1 is a cysteine residue bound through a thioester bond to a prenyl of 5 - 15 carbon atoms.

70. The method of claim 69, wherein X_1 is selected from the group consisting of myristic acid, palmitic acid and farnesyl cysteine.

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71. The method of claim 69, wherein the positively charged amino acid residues of X_3 are selected from the group consisting of lysine residue, arginine residue, histidine residue and an amino acid which includes a positively charged group covalently bound to an α-carbon atom or to an α-amine on a peptide backbone by a spacer chain of 1 - 4 carbon atoms, saturated or unsaturated, substituted or unsubstituted.

72. The method of claim 71, wherein the positively charged amino acid residues in X_3 are selected from the group consisting of lysine and arginine residues and combinations thereof.

73. The method of claim 68, wherein the polar uncharged amino acid residues of X_3 are selected from the group consisting of serine, threonine, asparagine and glutamine residues and combinations thereof.

74. The method of claim 68, wherein the aromatic amino acid residues of X_4 are selected from the group consisting of phenylalanine and tryptophan residues and combinations thereof.

75. The method of claim 68, wherein the polar uncharged amino acid residues of X_4 are selected from the group consisting of serine, asparagine and glutamine residues and combinations thereof.

76. The method of claim 68, wherein the hydrophobic aliphatic amino acid residues of X_4 are selected from the group consisting of leucine, alanine and glycine residues and combinations thereof.

77. The method of claim 68, wherein the anionic-phospholipid binding compound includes additional groups X_2, X_5 and X_6 and which has a general formula:

$X_1\ X_2\ [(X_3)_a / (X_4)_b /(X_5)_c]\ X_6$

wherein:

X_2 includes 0 - 3 glycine residues and 0 - 2 β-amino alanine molecules;

X_5 is a compound of a general formula:

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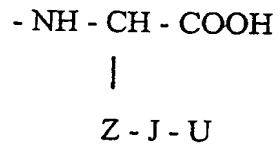

wherein:

Z is a spacer group containing a chain of 1 - 4 carbon atoms, saturated or unsaturated, substituted or unsubstituted;

J is a functional group selected from the group consisting of amines, thiols, alcohols, carboxylic acids, esters aldehydes and alkyl halides;

U is an anchoring group or said X_1 group;

c is an integer from 0 - 10; and

X_6 is zero or said X_1;

whereas, within subunit $[(X_3)_a/(X_4)_b/(X_5)_c]$ the groups X_3, X_4 and X_5 are arranged in one of all possible linear arrangements with respect to one another.

78. The method of claim 77, wherein X_5 is a lysine residue being substituted at an ε-amino group thereof by an anchoring group.

79. The method of claim 77, wherein said anionic-phospholipid binding compound is myristate-GGGKKKKKRFSFKKSFKLSGFSFKKNKKKU.

80. The method of claim 77, wherein said anionic-phospholipid binding compound is myristate-GGGKKKKKRFSFKKSFKLSGFSFKKNKKK(biotin).

81. The method of claim 77, wherein said anionic-phospholipid binding compound is myristate-KKKKKRFSFKKSFKLSGFSFKKNKKKU.

82. The method of claim 77, wherein said anionic-phospholipid binding compound is myristate-KKKKKRFSFKKSFKLSGFSFKKNKKK(biotin).

83. The method of claim 57, wherein said affinity filter forms a portion of an extracorporeal circulation apparatus.

84. The method of claim 57, wherein said affinity filter forms a portion of a blood

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transfusion apparatus.

85. The method of claim 57, wherein said affinity filter forms a portion of a hemodialysis apparatus.

86. The method of claim 57, wherein said affinity filter forms a portion of a plasmapheresis apparatus.

87. The method of claim 57, wherein the blood is of a patient selected from the group consisting of a patient undergoing a surgery, a patient undergoing hemodialysis, a patient undergoing a plasmapheresis, a diabetic patient, a thalassemic patient, a patient having systemic lupus erythematosus.

88. The method of claim 63, wherein said anionic-phospholipid binding compound is a compound of general formula III:

X_1-X_2-{$(X_3)_a$/X_4/X_5}-X_6

wherein:

X_1 stands for a saturated or unsaturated fatty acid residue comprising 6 - 20 carbon atoms; or a cysteine residue bound through a thioether bond to a prenyl group comprising 5 - 20 carbon atoms; the residue being linked to the adjacent component of the compound through an amide bond;

X_2 is either 0 or includes at least one unit of a general formula (IV) $A*2^n$, in which A represents a branching unit and *n* is an integer of 0 - 4;

X_3 comprises 1-6 amino acids, of which 1-6 are positively charged and 0-2 are negatively charged, any remaining amino acid residues being polar uncharged amino acids;

X_4 comprises 1-6 amino acids, of which 1-2 are aromatic amino acids, any remaining amino acids being selected among polar uncharged amino acids and hydrophobic aliphatic amino acids;

X_5 comprises 1-6 amino acids, of which 1-6 are positively charged and 0-2 are negatively charged, any remaining amino acid residues being polar uncharged amino acids, wherein the amino acids form a cyclic structure;

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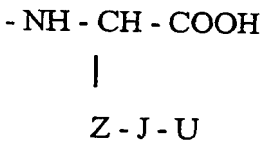
X_6 is selected from the group consisting of a linker and a compound of general formula II



wherein Z stands for a spacer group containing a chain of 1 - 5 carbon atoms, saturated or unsaturated, substituted or unsubstituted; J represents a functional group selected among amines, thiols, alcohols, carboxylic acids and esters, aldehydes and alkyl halides; and wherein: *a* is an integer of 1 – 3.

89. The method of claim 88, wherein said chain for Z is an alkane or an alkene.

90. The method of claim 88, wherein the groups X_3, X_4 and X_5 are each located at a separately selected location in the compound.

91. The method of claim 88, wherein X_1 is a residue of a saturated fatty acid of formula $CH_3(CH_2)_nCO_2H$, in which *n* is an integer of 8 - 18; or for a cysteine residue bound through a thioether bond to a prenyl group of 5 - 20 carbon atoms.

92. The method of claim 88, wherein X_1 is selected among myristic acid and palmitic acid; and farsenyl cysteine.

93. The method of any of claims 90 to 92, wherein branching unit A of X_2 is selected among a dicarboxylic and a poly-carboxylic acid.

94. The method of any of claims 90 to 93, wherein the positively charged amino acids of X_3 and X_5 are selected among lysine, arginine, histidine or any amino acid which is comprised of a positively charged group covalently bound to the α-carbon atom or to the α-amine on the peptide backbone by a spacer selected from a group comprised of an alkene of 1 - 5 carbon atoms and combinations thereof.

95. The method of claim 94, wherein the positively charged amino acids in X_3 and X_5 are selected among lysine and arginine and combinations thereof.

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96. The method of any of claims 90 to 95 wherein the negatively charged amino acids of X_3 and X_5 are selected among glutamate and aspartate.

97. The method of any of claims 90 to 96 wherein the polar uncharged amino acids of X_3 and X_5 are selected among serine, threonine, asparagine and glutamine and combinations thereof.

98. The method of any of claims 89 to 97, wherein the aromatic acids of X_4 are selected among phenylalanine and tryptophan and combinations thereof.

99. The method of any of claims 90 to 98, wherein the polar uncharged amino acids of X_4 are selected among serine, asparagine and glutamine and combinations thereof.

100. The method of any of claims 90 to 99, wherein the hydrophobic aliphatic amino acids of X_4 are selected among leucine, valine, alanine and glycine and combinations thereof.

101. The method of any of claims 88 to 100, wherein the cyclization of X_5 is performed via an intra-molecular di-sulfide bridge or by an amide bond.

102. The method of claim 101, wherein X_6 stands for a cysteine residue bound through a thioether bond to a prenyl group, in which the cysteine carboxyl is either free or methylated.

103. The method of claim 88, wherein said compound of general formula III is Myristate - KVSFFCKNKEKKC - K.

104. The method of claim 88, wherein said compound of general formula III is Myristate - KVSFFCKNKEKKC – K and wherein the two cysteine residues are linked via a disulfide bond, to form a cyclic structure.

105. The method of claim 88, wherein said compound of general formula III isMyristate - KKVFSFCKNKEKKC- K.

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106. The method of claim 88, wherein said compound of general formula III is Myristate - KKVFSFCKNKEKKC – K, wherein the two cysteine residues are linked together.

107. The method of any of claims 103 to 105, wherein said compound of general formula III further features said linker for linking said compound of general formula III to said solid support.

108. The method of claim 107, wherein said linker is βA-βA-P-βA-βA.

109. A compound, comprising a compound of general formula III:

X_1-X_2-{$(X_3)_a$/X_4/X_5}-X_6

wherein:

X_1 stands for a saturated or unsaturated fatty acid residue comprising 6 - 20 carbon atoms; or a cysteine residue bound through a thioether bond to a prenyl group comprising 5 - 20 carbon atoms; the residue being linked to the adjacent component of the compound through an amide bond;

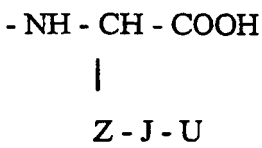
X_2 is either 0 or includes at least one unit of a general formula (IV) A^*2^n, in which A stands for a branching unit and n stands for 0 - 4;

X_3 comprises 1-6 amino acids, of which 1-6 are positively charged and 0-2 are negatively charged, any remaining amino acid residues being polar uncharged amino acids;

X_4 comprises 1-6 amino acids, of which 1-2 are aromatic amino acids, any remaining amino acids being selected among polar uncharged amino acids and hydrophobic aliphatic amino acids;

X_5 comprises 1-6 amino acids, of which 1-6 are positively charged and 0-2 are negatively charged, any remaining amino acid residues being polar uncharged amino acids, wherein the amino acids form a cyclic structure;

X_6 is selected from the group consisting of a linker, and a compound of general formula II



wherein Z stands for a spacer group containing a chain of 1 - 5 carbon atoms, saturated or unsaturated, substituted or unsubstituted, preferably alkane or alkene; J represents a functional

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group selected among amines, thiols, alcohols, carboxylic acids and esters, aldehydes and alkyl halides; U is either 0 or a labeling moiety; wherein: *a* is an integer of 1 – 3.

110. The compound of claim 109, wherein the groups X_3, X_4 and X_5 are each located at a separately selected location in the compound.

111. The compound of claim 109, wherein X_1 is a residue of a saturated fatty acid of formula $CH_3(CH_2)_nCO_2H$, in which *n* stands for an integer of 8 - 18; or for a cysteine residue bound through a thioether bond to a prenyl group of 5 - 20 carbon atoms.

112. The compound of claim 109, wherein X_1 is selected among myristic acid, palmitic acid and farsenyl cysteine.

113. The compound of claims 109 to 112, wherein branching unit A of X_2 is selected from the group consisting of a dicarboxylic acid and a poly-carboxylic acid.

114. The compound of any of claims 109 to 113, wherein the positively charged amino acids of X_3 and X_5 are selected among lysine, arginine, histidine or any amino acid which is comprised of a positively charged group covalently bound to the α-carbon atom or to the α-amine on the peptide backbone by a spacer containing a carbon chain of 1 - 4 carbon atoms including an alkene.

115. The compound of claim 114, wherein the positively charged amino acids in X_3 and X_5 are selected among lysine and arginine and combinations thereof.

116. The compound of any of claims 109 to 115, wherein the negatively charged amino acids of X_3 and X_5 are selected among glutamate and aspartate.

117. The compound of any of claims 109 to 116, wherein the polar uncharged amino acids of X_3 and X_5 are selected among serine, threonine, asparagine and glutamine and combinations thereof.

118. The compound of any of claims 109 to 117, wherein the aromatic acids of X_4 are

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selected among phenylalanine and tryptophan and combinations thereof.

119. The compound of any of claims 109 to 118, wherein the polar uncharged amino acids of X_4 are selected among serine, asparagine and glutamine and combinations thereof.

120. The compound of any of claims 109 to 119, wherein the hydrophobic aliphatic amino acids of X_4 are selected among leucine, valine, alanine and glycine and combinations thereof.

121. The compound of any of claims 109 to 120, wherein the cyclization of X_5 is performed via an intra-molecular di-sulfide bridge or by an amide bond or via a bond to ^{99}Tc.

122. The compound of any of claims 109 to 121, wherein U is a labeling group for specific binding selected among biotin and a group containing a substituent selected among a fluorescein, a radio-isotope and a paramagnetic contrast agent.

123. The compound of claim 122, wherein the fluorescein is fluorescein isothiocyanate.

124. The compound of claim 122, wherein the radioisotope is selected among technetium, lead, mercury, thallium and indium.

125. The compound of claim 122, wherein the paramagnetic contrast agent is a paramagnetic metal ion chelate.

126. The compound of any of claims 109 to 125, wherein X_6 is a lysine residue being substituted at the ε-amino group by a labeling group according to any of claims 109 and 122 to 125.

127. The compound of any of claims 109 to 126, wherein X_6 stands for a cysteine residue bound through a thioether bond to a prenyl group, in which the cysteine carboxyl is either free or methylated.

128. The compound of claim 109, wherein said compound is

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Myristate - KVSFFCKNKEKKC - KU, wherein U is as defined in any of claims 109 and 122 to 125.

129. The compound of claim 109, wherein said compound is

Myristate - KVSFFCKNKEKKC - K(biotin) in which the two cysteine residues are linked via a disulfide bond, to form a cyclic structure.

130. The compound of claim 109, wherein said compound is

Myristate - KKVFSFCKNKEKKC- KU, wherein U is as defined in any of claims 107 and 120 to 123.

131. The compound of claim 109, wherein said compound is

Myristate - KKVFSFCKNKEKKC - K(biotin) wherein the two cysteine residues are linked together.

132. The compound of any of claims 128 to 131, wherein said compound of general formula III further features said linker for linking said compound of general formula III to said solid support.

133. The compound of claim 132, wherein said linker is βA-βA-P-βA-βA.

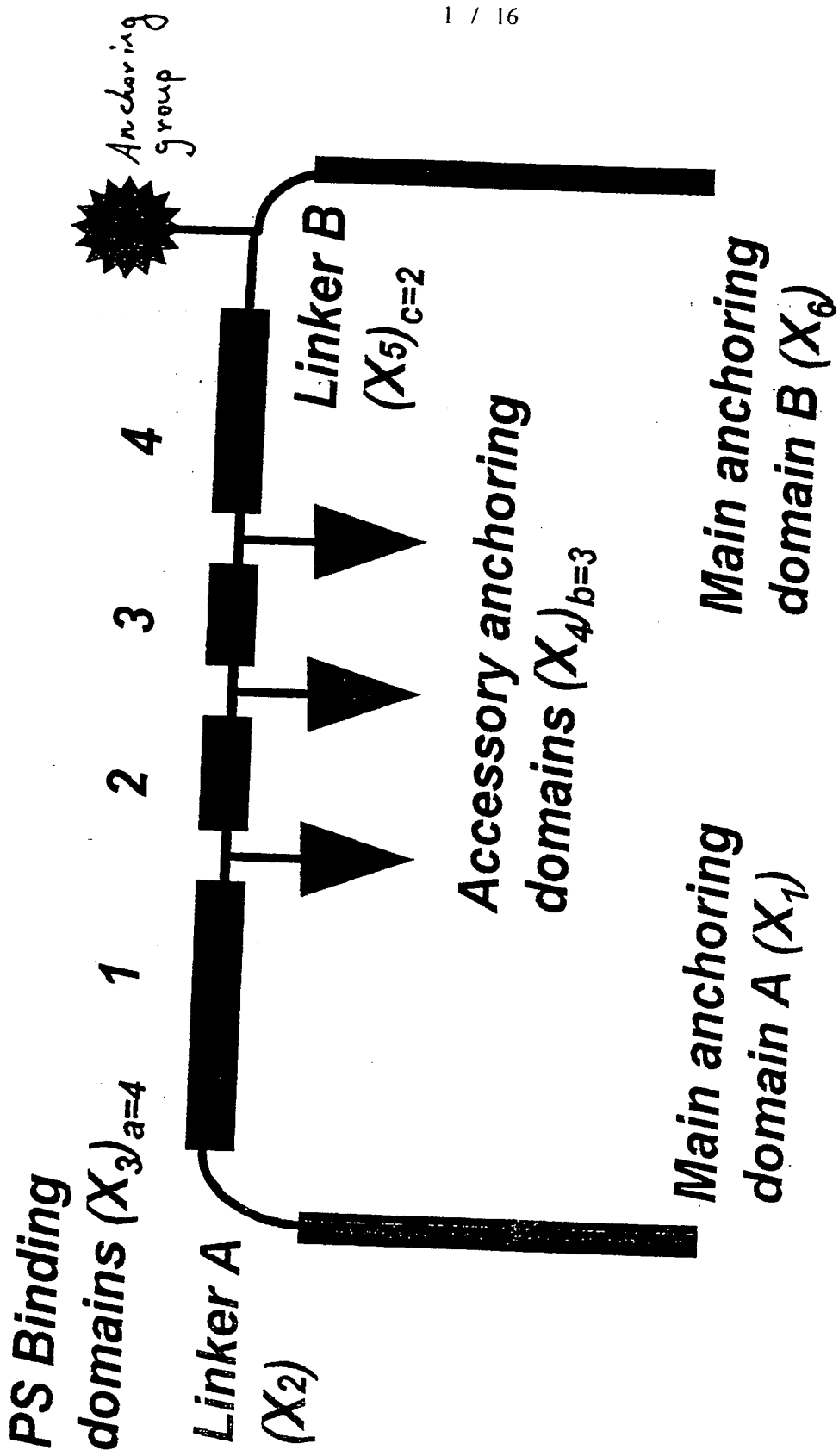

Fig. 1a

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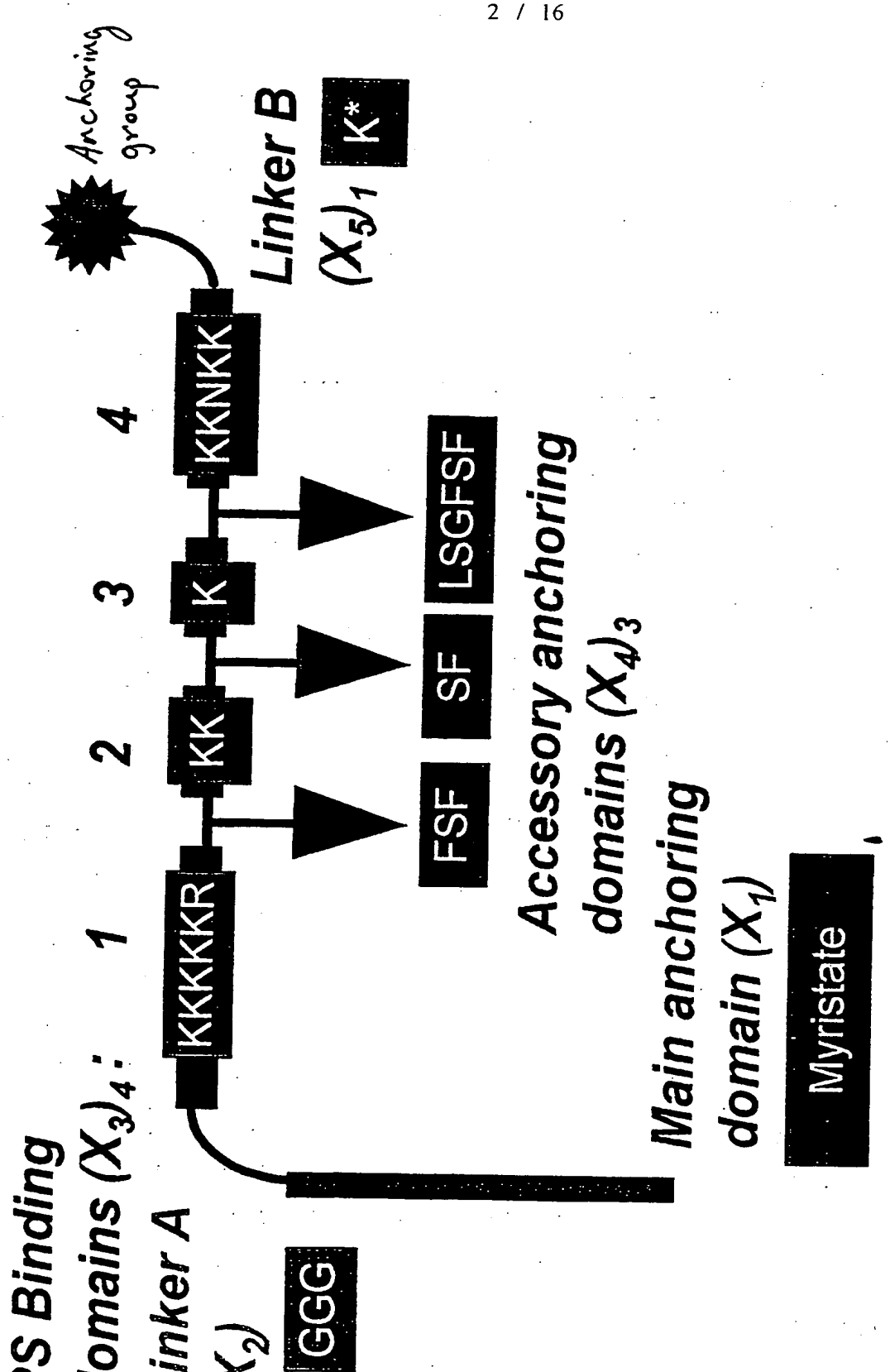

Fig. 1b

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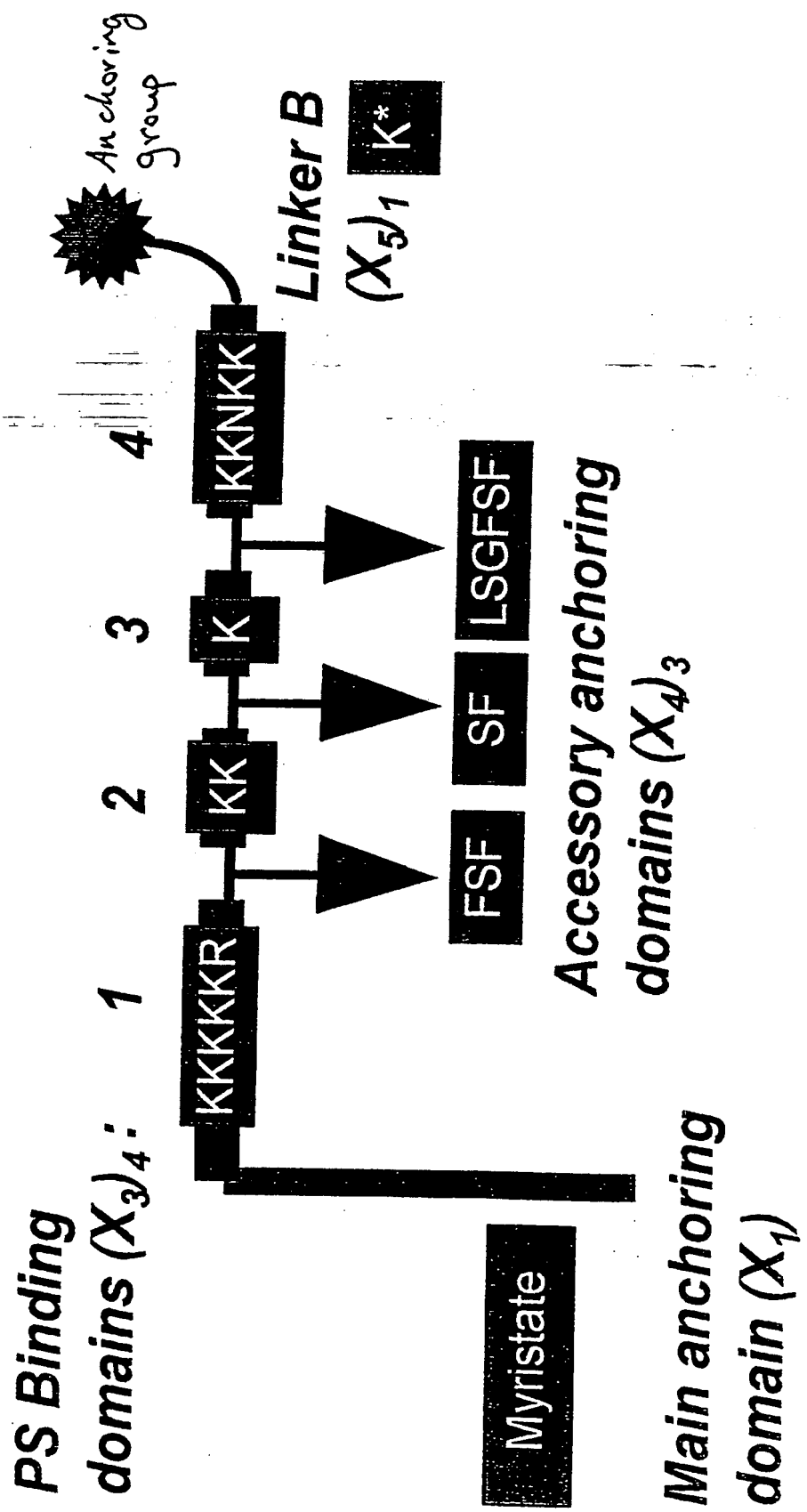

Fig. 1c

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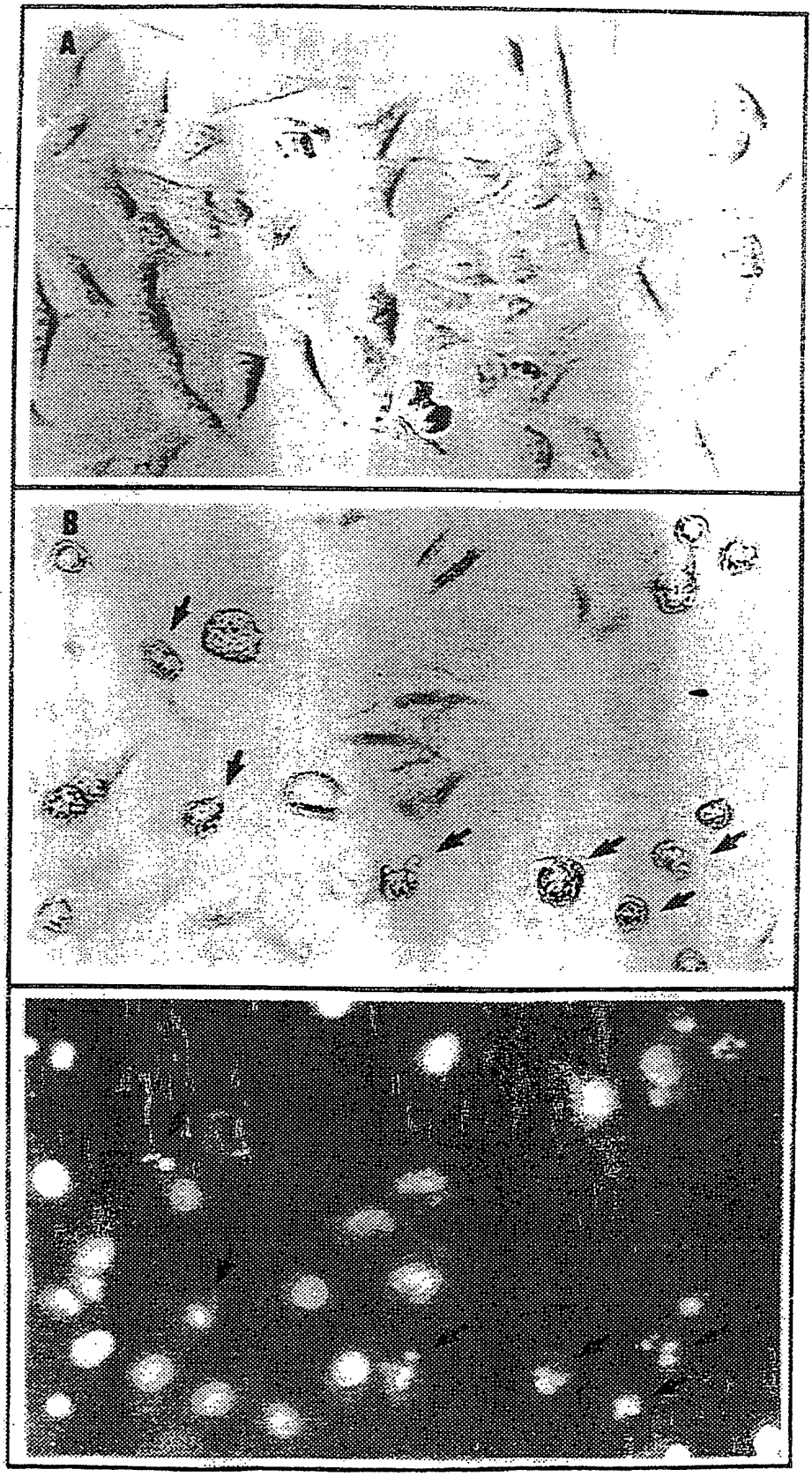
Figure 2

Cultured Hela cells undergoing DA-induced apoptosis



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Detection of apoptosis by NST301 compound

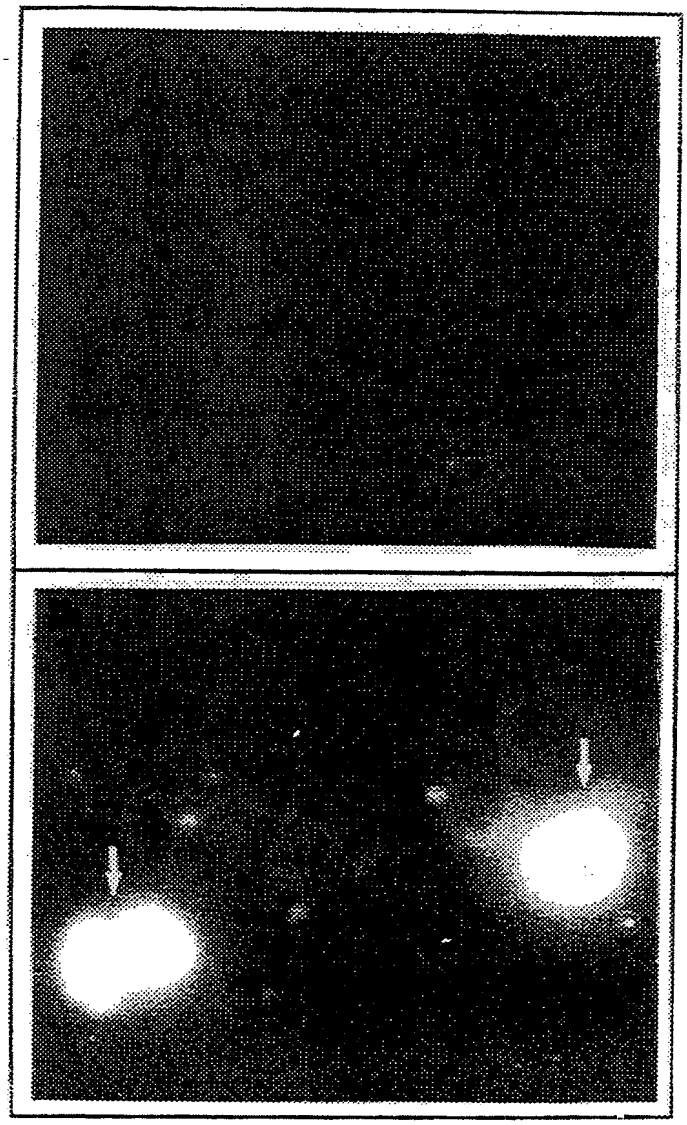

Fig. 2d

Fig. 2e

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Detection of apoptotic cells by NST301 compound

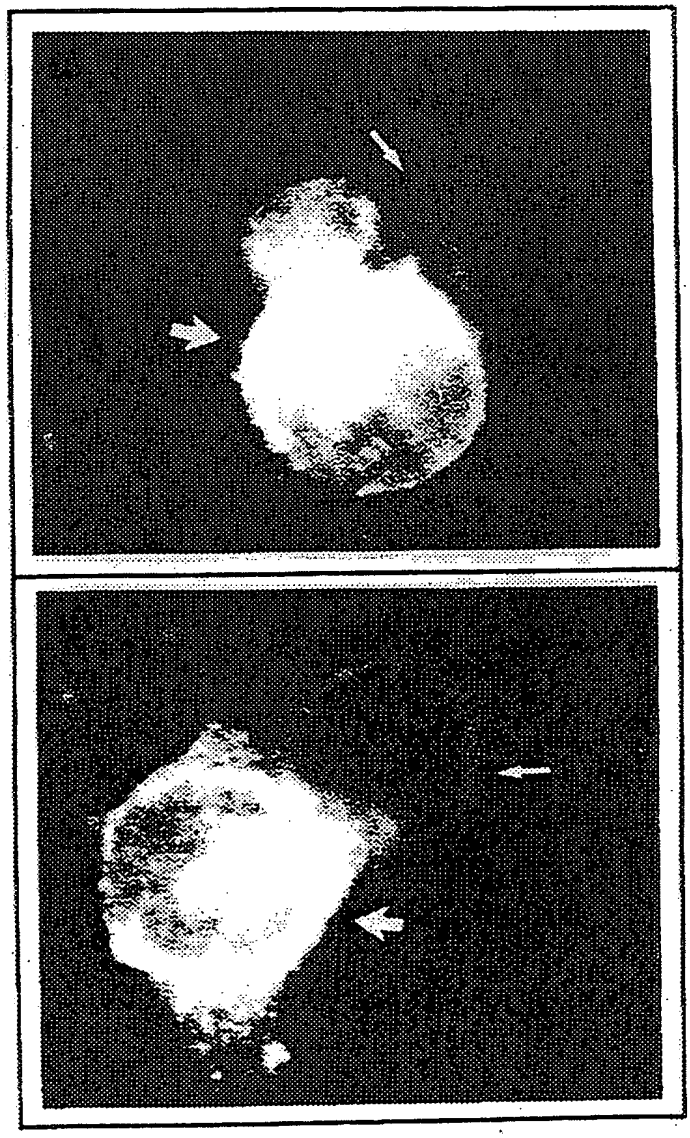

Fig. 2f

Fig. 2g

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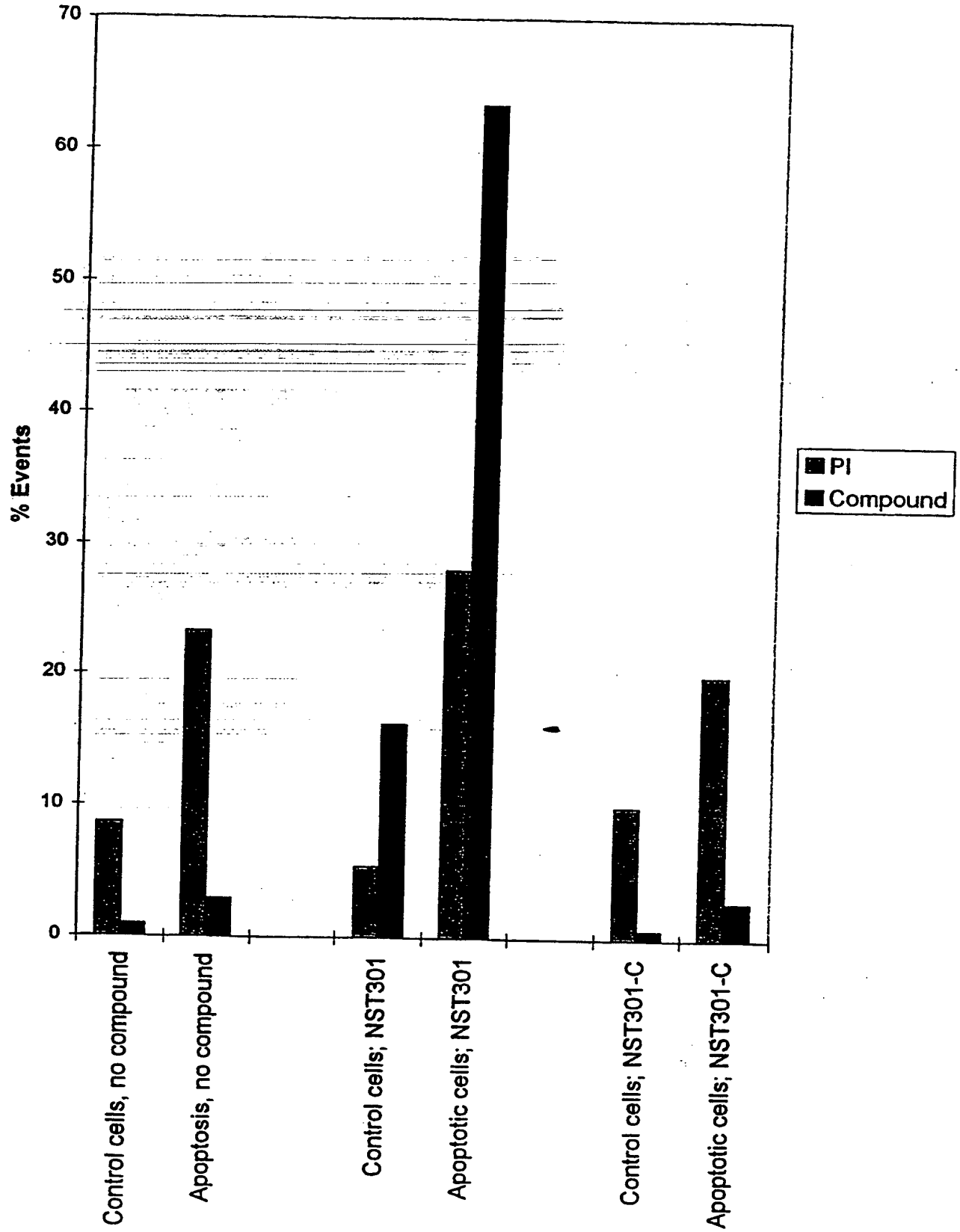
70
60
50
40
30
20
10
0
% Events
PI
Compound
Control cells, no compound
Apoptosis, no compound
Control cells; NST301
Apoptotic cells; NST301
Control cells; NST301-C
Apoptotic cells; NST301-C

Fig. 3a

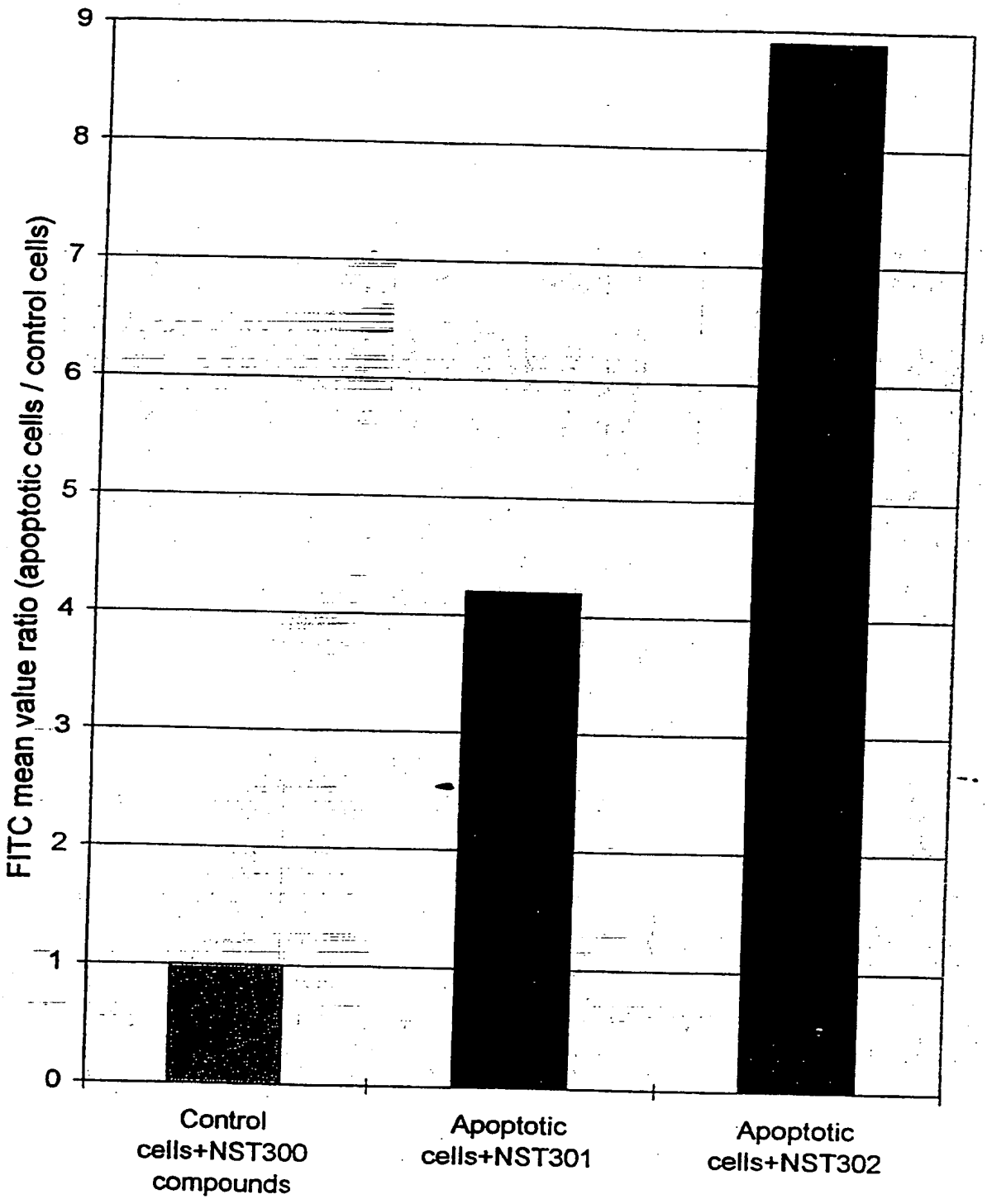
FITC mean value ratio (apoptotic cells / control cells)
0
1
2
3
4
5
6
7
8
9
Control cells+NST300 compounds
Apoptotic cells+NST301
Apoptotic cells+NST302

Fig. 3b

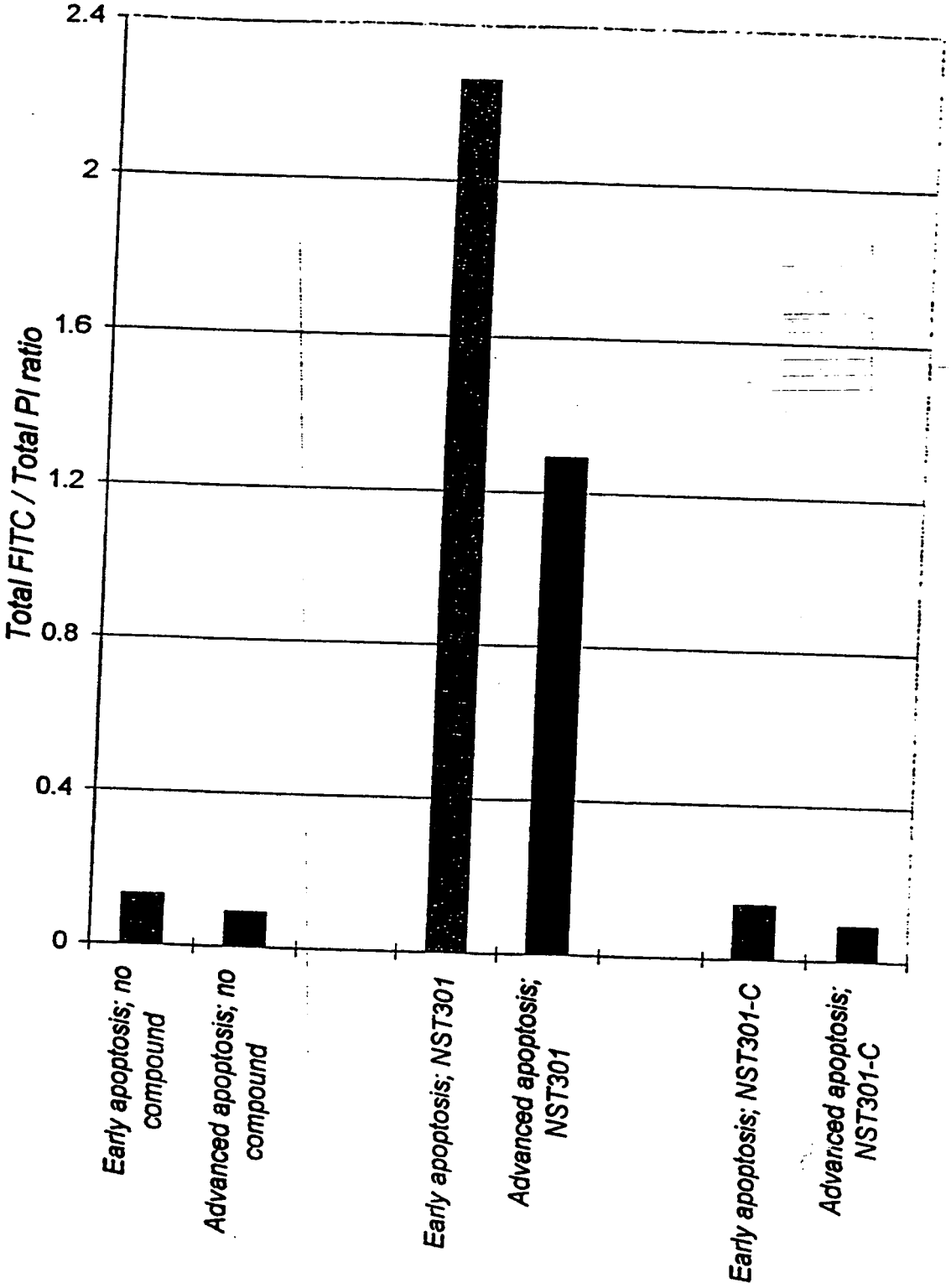

Fig. 3c

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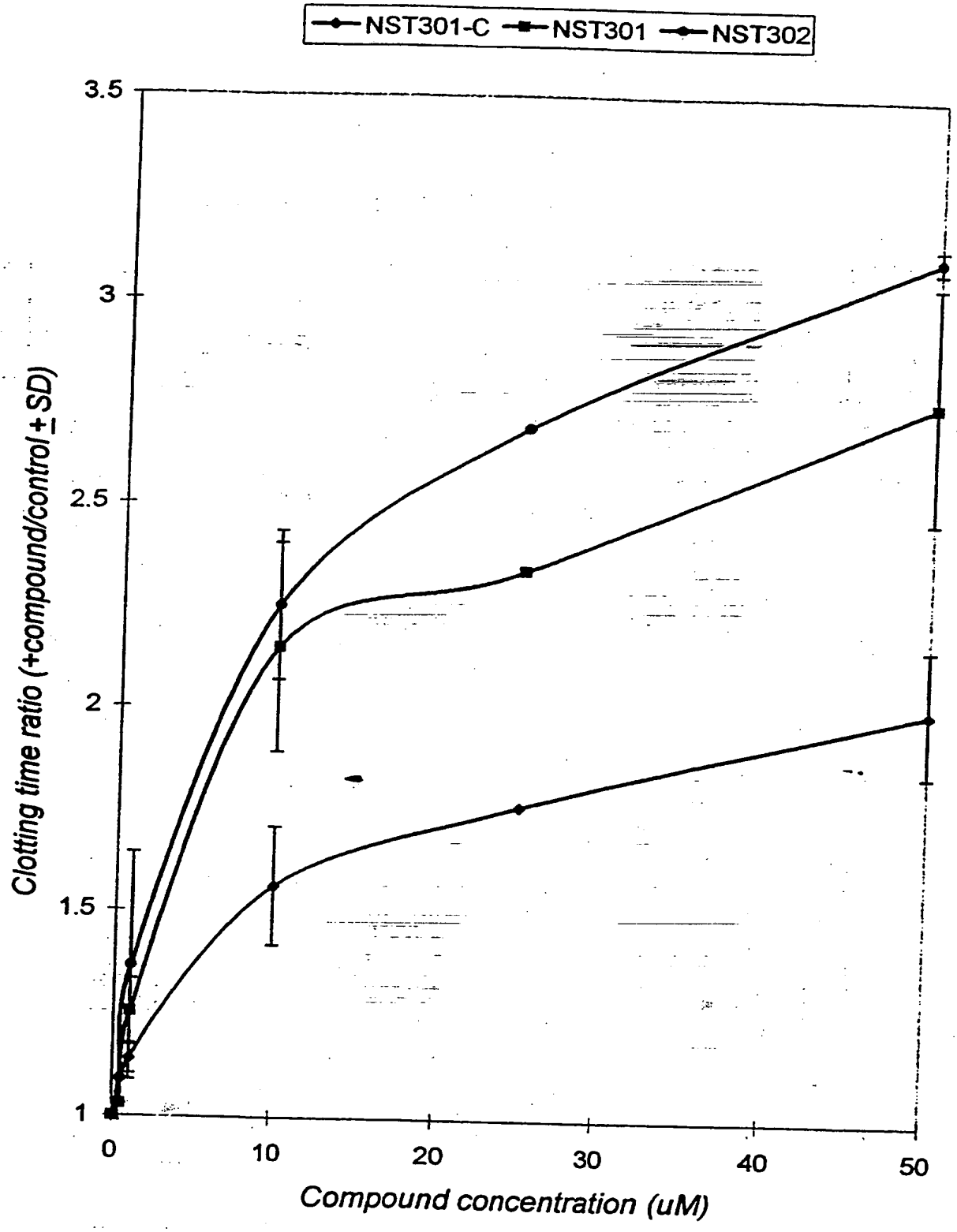

Fig. 4

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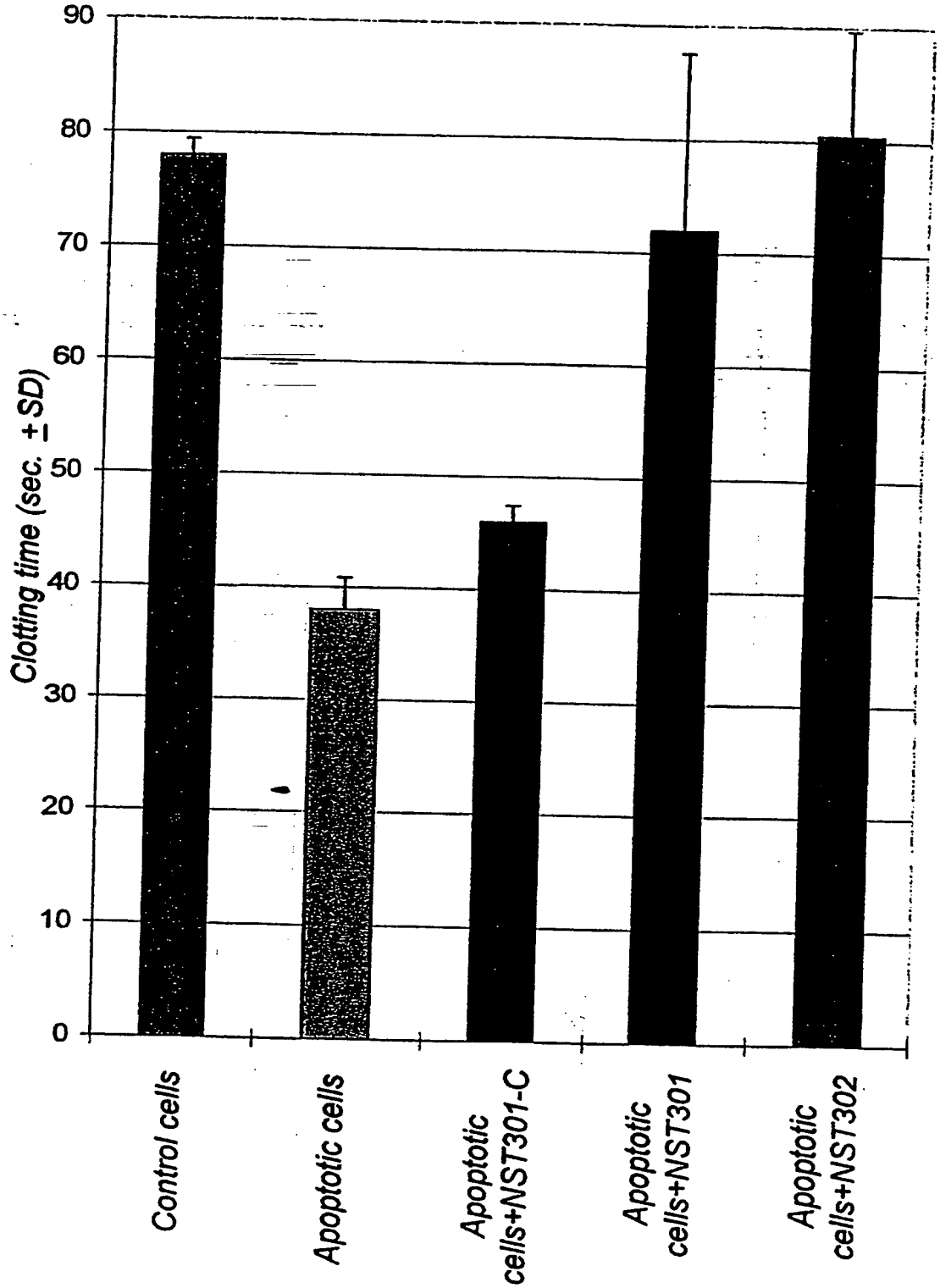

Fig. 5

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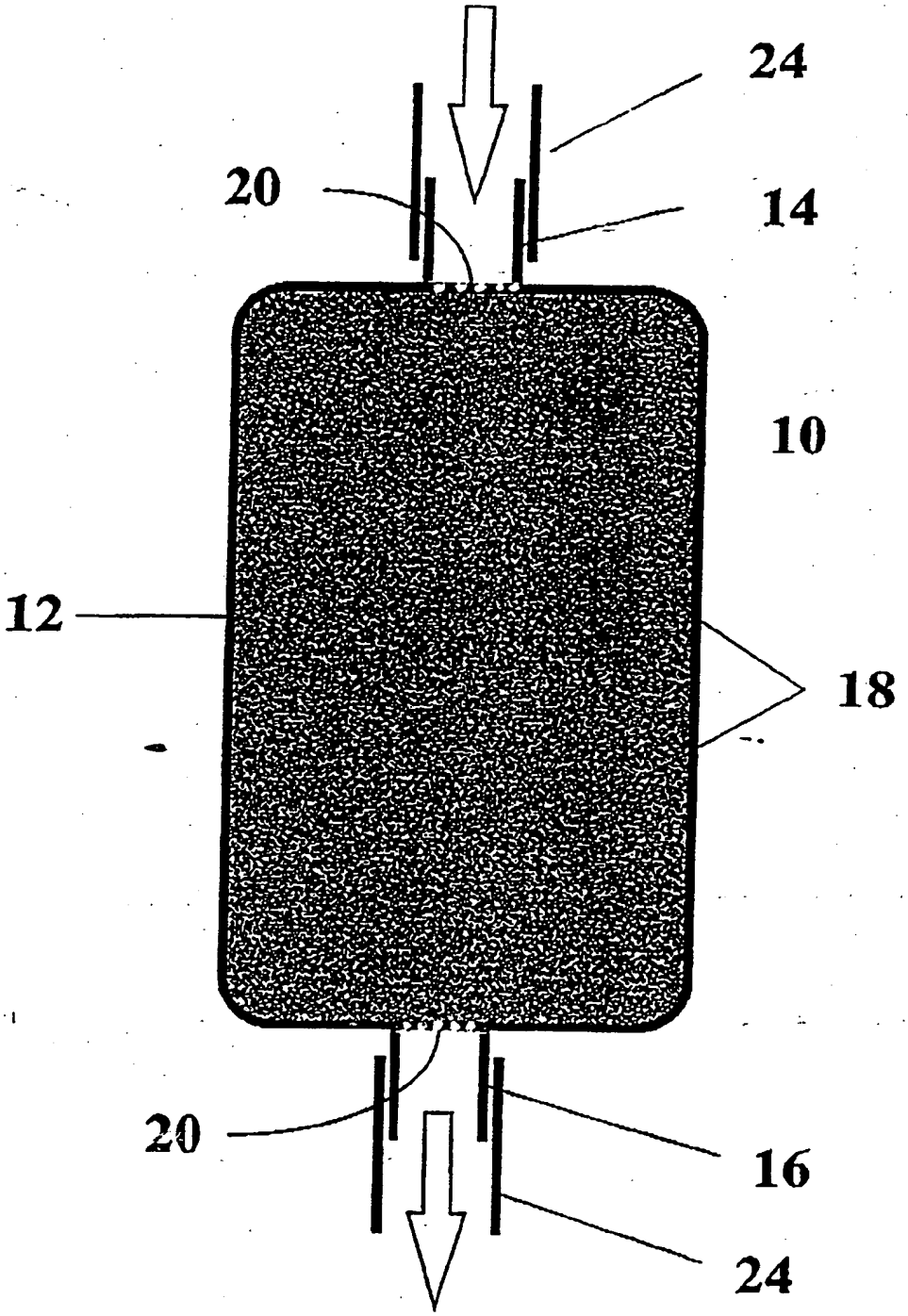

Fig. 6

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Binding of apoptotic cells to NST302-coated beads: fluorescent microscopy

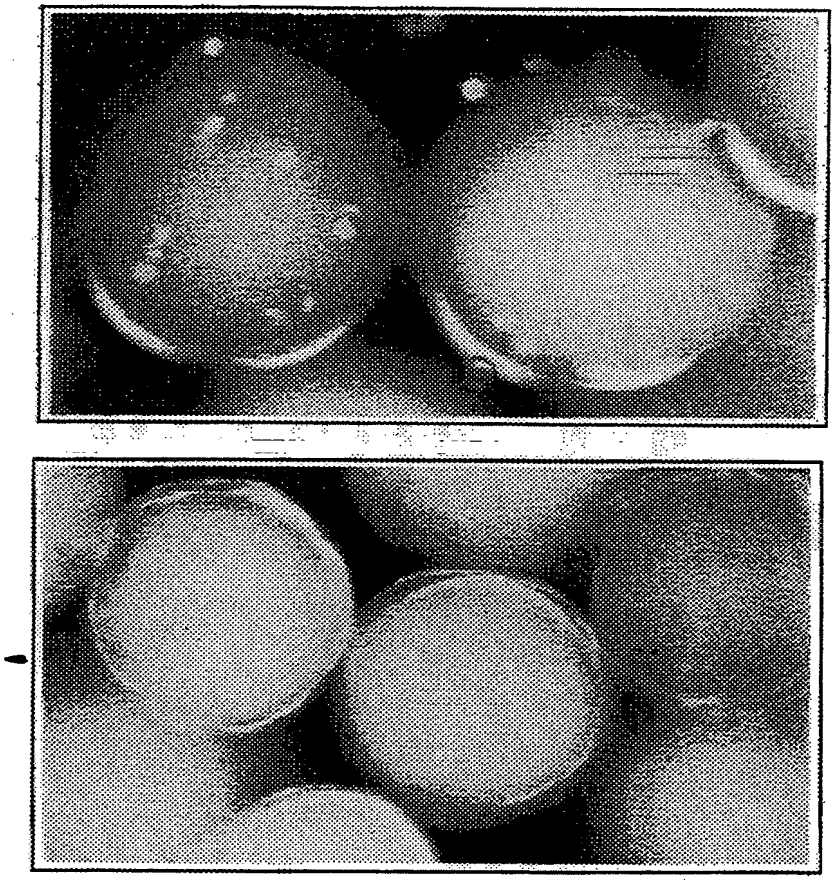

Fig. 7: Fluorescent microscopy of B-302 (upper) and control beads (lower) after incubation with apoptotic HeLa cells and staining with Hoechst 33342.

Binding of apoptotic cells to NST302-coated beads: confocal microscopy

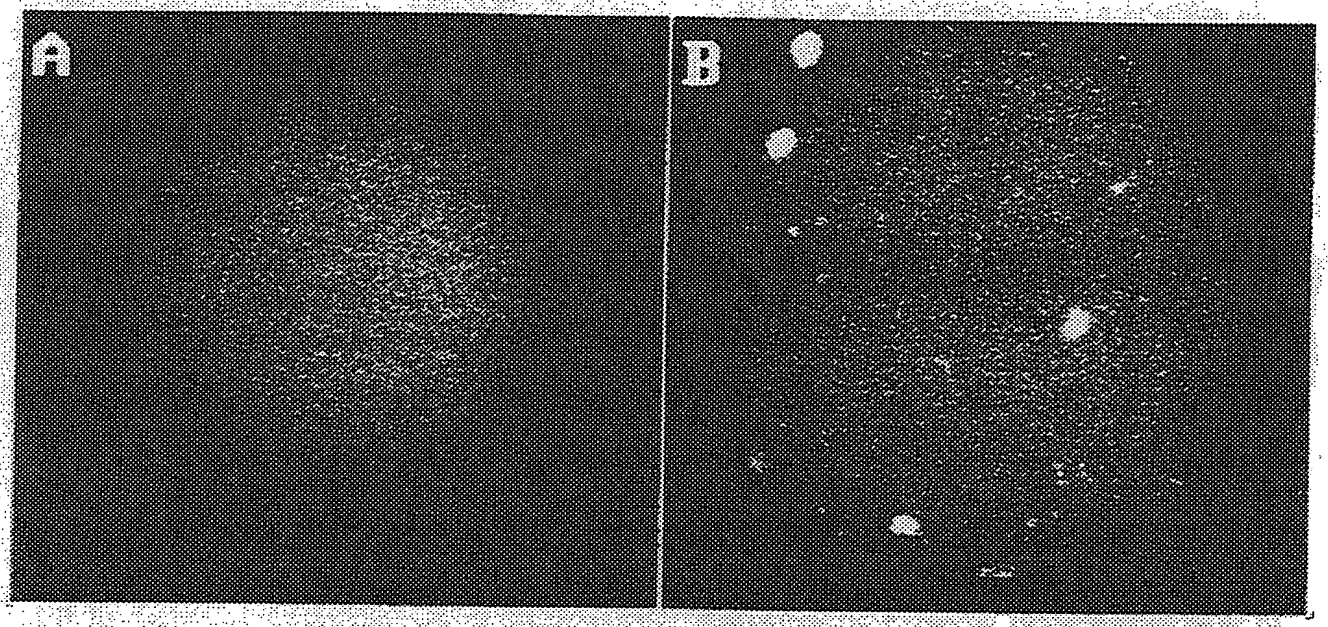

Fig. 8: Confocal microscopy of B-302 (left) and control beads (right) after incubation with apoptotic HeLa cells and staining with Hoechst 33342.

Treatment of apoptotic HeLa cells with NST302-coated beads reduces their procoagulant activity.

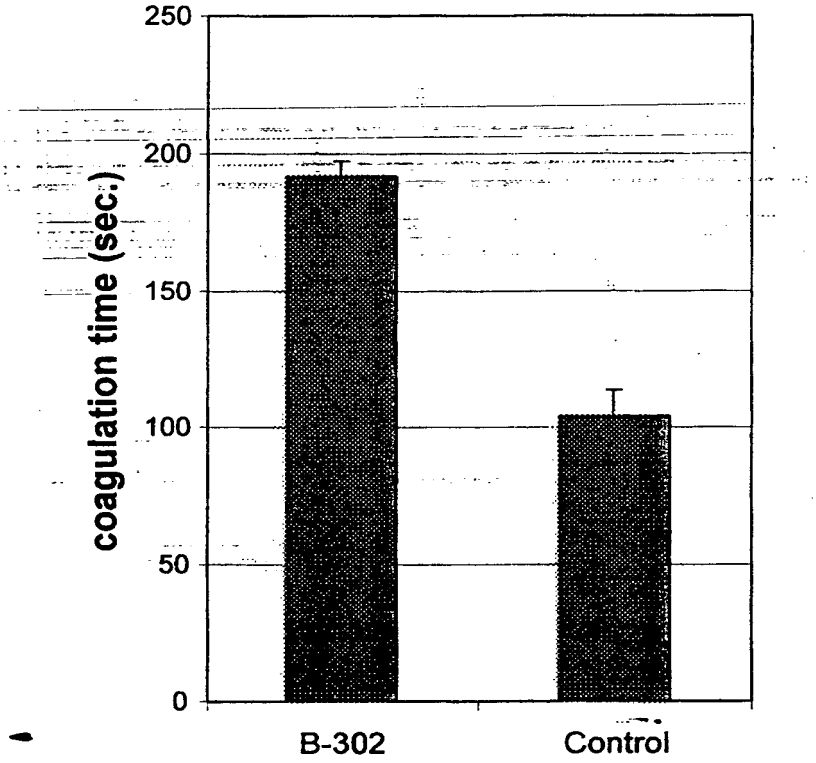

Fig. 9: Treatment of apoptotic HeLa cells with B-302 increases coagulation time. Equal number of cells treated with either B-302 or control beads were used to measure coagulation time in a modified APTT test.

NST513-coated beads selectively bind activated platelet

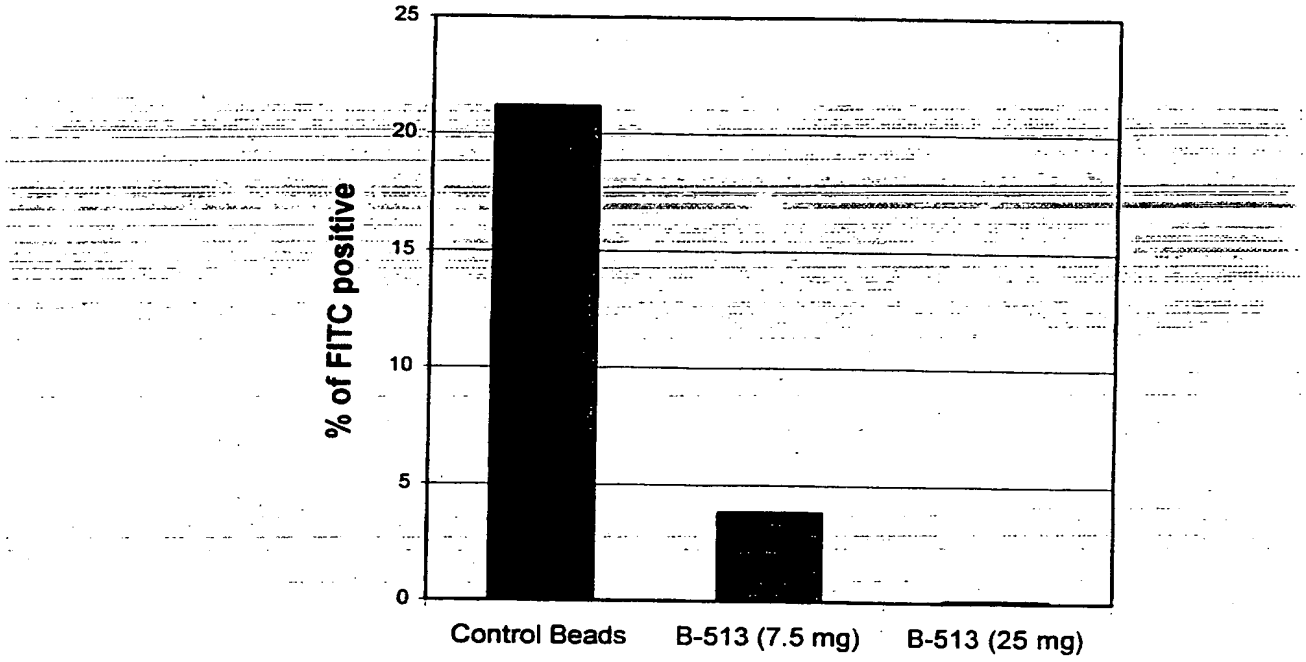

Fig. 10: Platelets were washed in platelets buffer and activated with the Ca ionophore A23187 in the presence of $CaCl_2$. Activated and control platelets were mixed in 1:1 ratio and were incubated with Annexin V-biotin following labeling with Strepavidin-FITC. The washed platelets were subjected to FACS analysis and % FITC positive was determined according to the level of labeling on activated vs. control platelets.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL99/00459

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :B01D 11/00, 19/02, 21/24, 53/22; B01J 11/00; A01N 1/02; C12Q 1/58

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 96/6, 178, 179; 210/98, 646, 782, 805; 422/ 44, 48; 435/ 2, 13; 436/66; 604/4, 7, 8, 28, 48

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, BIOSIS, EMBASE, DIALOG, CAPLUS, WPIDS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,630,946 A (HART et al) 20 May 1997, columns 1-12.	1-7, 9-12, 30, 56-67 and 87
Y	EP 0 755 516 B2 (NEXINS RESEARCH B.V.) 29 January 1997, columns 1-7.	1-7, 9-12, 30, 56-67 and 87
Y	US 3,701,433 A (KRAKAUER et al) 31 October 1972, columns 1-2.	8, 31, 62, and 83
Y	US 4,350,594 A (KAWAI et al) 21 September 1982, columns 1-2.	32, 33 and 84 -86
A	US 5,567,615 A (DEGEN et al) 22 October 1996, see whole document.	1-12, 30-34, 56-67 and 83-87

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
20 NOVEMBER 1999

Date of mailing of the international search report
01 FEB 2000

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL99/00459

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,744,047 A (GSELL et al) 28 April 1998, see whole document.	1-12, 30-33, 56-67 and 83-87
A	WO 95/34315 A1 (NEORX CORPORATION) 21 December 1995, see whole document.	1-12, 30-33, 56-67 and 83-87
A	US 4,572,724 A (ROSENBERG et al) 25 February 1986, see whole document.	1-12, 30-33, 56-67 and 83-87

BNSDOCID: <WO___0010673A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL99/00459

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 13-29, 34-55, 68-82 and 88-133
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

These claims could not be searched because it is impossible to determine how the structural portions are connected based upon the notation of the general formula provided. Further these claims teach sequences, without complying to the sequence rules required.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL99/00459

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

96/6, 178, 179; 210/98, 646, 782, 805; 422/44, 48; 435/2, 13; 436/66; 604/4, 7, 8, 28, 48

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
B01D 11/00, 19/02, 21/24, 53/22,
A01N 1/02, C12Q 1/58

A1

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With a revised version of the international search report.

(88) Date of publication of the revised version of the international search report: 27 July 2000 (27.07.00)

(54) Title: APPARATUS AND METHOD FOR CAPTURING PARTICLES WITH SURFACE EXPOSURE OF ANIONIC PHOSPHOLIPIDS FROM BIOLOGICAL FLUIDS

(57) Abstract

An affinity filter and method of using same, effective in capturing and thereby removing particles characterized by surface exposure of anionic phospholipids present in a biological fluid, particularly blood or blood–derived products. Examples of other biological fluids include semen, cerebrospinal fluid, urine and mucous. The affinity filter includes a body formed with an inlet and an outlet, including a solid support and an anionic–phospholipid binding compound linked to the solid support. The positively charged anionic–phospholipid binding compound serves for specifically binding the particles characterized by surface exposure of anionic phospholipids and thereby removing the particles from the biological fluid, and particularly from blood or blood–derived products, for example in order to prepare the blood or blood–derived product for transfusion into a subject.

*(Referred to in PCT Gazette No. 30/2000, Section II)

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL99/00459

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :B01D 11/00, 19/02, 21/24, 53/22; A01N 1/02; C12Q 1/58

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 96/6, 178, 179; 210/98, 646, 782, 805; 422/ 44, 48; 435/ 2, 13; 436/66; 604/4, 7, 8, 28, 48

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, BIOSIS, EMBASE, DIALOG, CAPLUS, WPIDS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,630,946 A (HART et al) 20 May 1997, columns 1-12.	1-7, 9-12, 30, 56-67 and 87
Y	EP 0 755 516 B2 (NEXINS RESEARCH B.V.) 29 January 1997, columns 1-7.	1-7, 9-12, 30, 56-67 and 87
Y	US 3,701,433 A (KRAKAUER et al) 31 October 1972, columns 1-2.	8, 31, 62, and 83
Y	US 4,350,594 A (KAWAI et al) 21 September 1982, columns 1-2.	32, 33 and 84 -86
A	US 5,567,615 A (DEGEN et al) 22 October 1996, see whole document.	1-12, 30-34, 56-67 and 83-87

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"O"	document referring to an oral disclosure, use, exhibition or other means		
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Date of the actual completion of the international search 20 NOVEMBER 1999	Date of mailing of the international search report 12 APR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JA-NA HINES Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
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